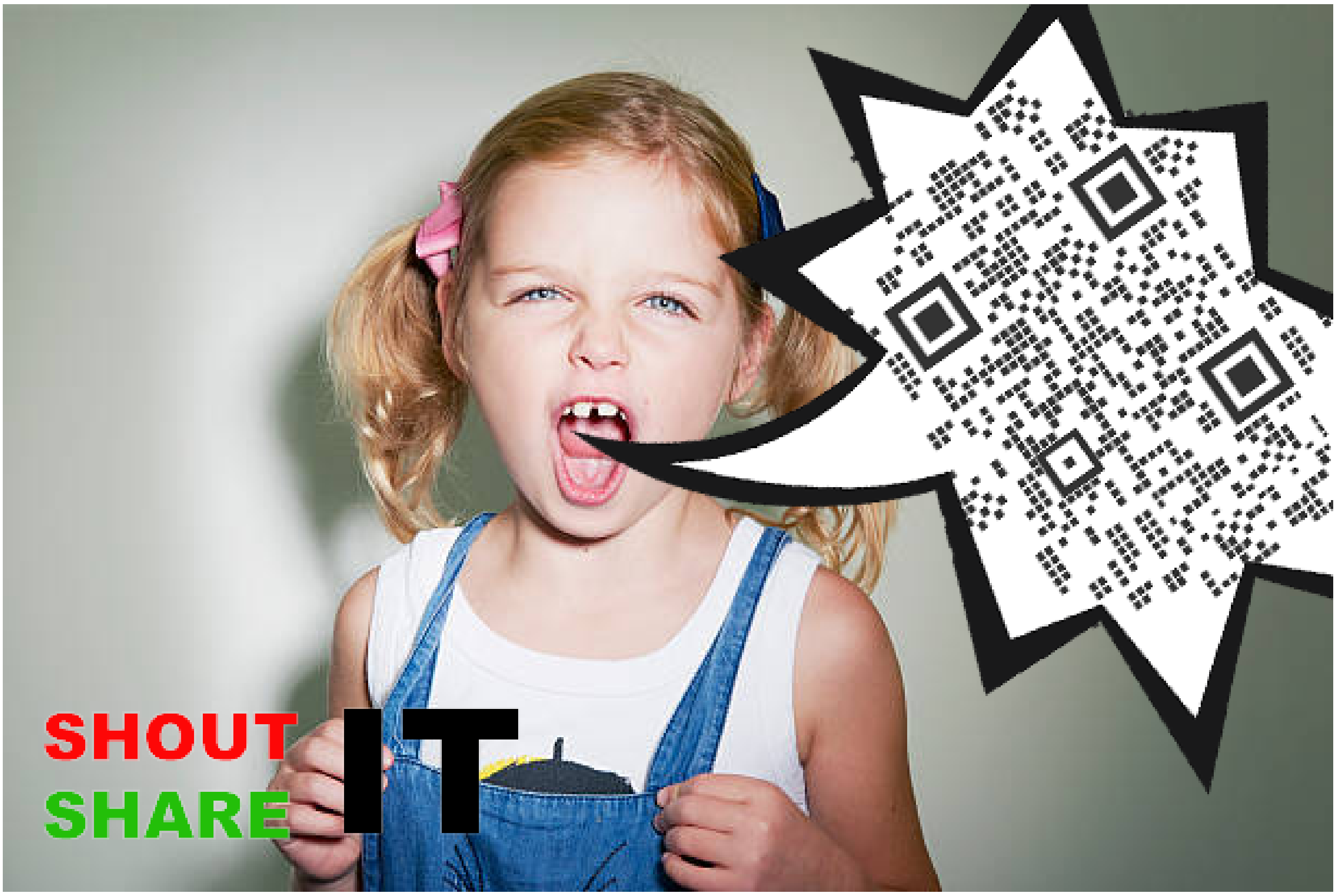

SHOUT
SHARE
IT

THE

BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

JOHN M. ANDERSON, Cornell University
JOHN B. BUCK, National Institute of Health
JOHN O. CORLISS, University of Maryland
JOHN D. COSTLOW, Duke University
CATHERINE HENLEY, University of North Carolina
GEORGE O. MACKIE, University of Victoria
F. H. RUDDLE, Yale University
BERTA SCHARRER, Albert Einstein College of Medicine
HOWARD A. SCHNEIDERMAN, University of California, Irvine
GROVER C. STEPHENS, University of California, Irvine
CARROLL M. WILLIAMS, Harvard University
EDWARD O. WILSON, Harvard University

W. D. RUSSELL-HUNTER, Syracuse University
Managing Editor

VOLUME 148

JANUARY TO JUNE, 1975

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $5.00. Subscription per volume (three issues), $14.00.

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between May 23 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

Second-class postage paid at Lancaster, Pa.

LANCASTER PRESS, INC., LANCASTER, PA.

CONTENTS

No. 1, February, 1975

Betzer, Susan B. and Michael E. Q. Pilson
Copper uptake and excretion by *Busycon canaliculatum* L. 1

Betzer, Susan B. and Paul P. Yevich
Copper toxicity in *Busycon canaliculatum* L. 16

Bradley, Brian P.
The anomalous influence of salinity on temperature tolerances of summer and winter populations of the copepod *Eurytemora affinis* 26

Bush, Louise
Biology of *Neochildia fusca* n. gen., n. sp. from the northeastern coast of the United States (Platyhelminthes: Turbellaria) 35

Komatsu, Miéko
On the development of the sea-star, *Astropecten latespinosus* Meissner .. 49

Kruczynski, W. L.
A radioactive tracer study of food uptake by *Pinnotheres maculatus* in molluscan hosts 60

Polls, Irwin and J. Gonor
Behavioral aspects of righting in two asteroids from the Pacific Coast of North America 68

Roberts, John L.
Active branchial and ram gill ventilation in fishes 85

Sehnal, Frantisek and Noelle A. Granger
Control of corpora allata function in larvae of *Galleria mellonella* 106

Shick, J. Malcolm
Uptake and utilization of dissolved glycine by *Aurelia aurita* scyphistomae: temperature effects on the uptake process; nutritional role of dissolved amino acids 117

Stefano, George B. and Edward Aiello
Histofluorescent localization of serotonin and dopamine in the nervous system and gill of *Mytilus edulis* (Bivalvia) 141

Webster, Steven K.
Oxygen consumption in echinoderms from several geographical locations, with particular reference to the Echinoidea 157

WEBSTER, STEVEN K. AND ARTHUR C. GIESE
Oxygen consumption of the purple sea urchin with special reference to the reproductive cycle.......... 165

NO. 2, APRIL, 1975

BLANQUET, RICHARD S. AND BRUCE WETZEL
Surface ultrastructure of the Scyphopolyp, *Chrysaora quinquecirrha*.... 181

DANIELS, BRUCE A. AND ROY T. SAWYER
The biology of the leech *Myzobdella lugubris* infesting blue crabs and catfish.......... 193

DUNN, DAPHNE FAUTIN
Reproduction of the externally brooding sea anemone *Epiactis prolifera* Verrill, 1869.......... 199

HOLLAND, NICHOLAS D., JOHN C. GRIMMER AND HIROSHI KUBOTA
Gonadal development during the annual reproductive cycle of *Comanthus japonica* (Echinodermata: Crinoidea).......... 219

KECK, RICHARD T., DON MAURER AND HENRY LIND
A comparative study of the hard clam gonad developmental cycle.... 243

KUNKEL, J. G.
Cockroach molting. I. Temporal organization of events during the molting cycle of *Blattella germanica* (L.).......... 259

MILKMAN, ROGER
Specific death sites in a *Drosophila* population cage.......... 274

RAVINDRANATH, M. H.
Effects of temperature on the morphology of hemocytes and coagulation process in the mole-crab *Emerita* (=*Hippa*) *asiatica*.......... 286

ROBERTSON, JAMES D.
Osmotic constituents of the blood plasma and parietal muscle of *Squalus acanthias* L.......... 303

SLÁMA, KAREL AND MAGDALENA HODKOVÁ
Insect hormones and bioanalogues: their effect on respiratory metabolism in *Dermestes vulpinus* L. (Coleoptera).......... 320

SULKIN, STEPHEN D.
The influence of light in the depth regulation of crab larvae.......... 333

WERMUTH, JEROME F. AND CHARLES D. BARNES
Dose-response effects of gamma-radiation on several growth functions of *Campanularia flexuosa*.......... 344

NO. 3, JUNE, 1975

ANDERSON, ROBERT S. AND ROBERT A. GOOD
Naturally-occurring hemagglutinin in a tunicate *Halocynthia pyriformis* 357

BROWN, FRANK A., JR. AND CAROL S. CHOW
Non-equivalence for bean seeds of clockwise and counterclockwise magnetic motion: a novel terrestrial adaptation? 370

CARR, WILLIAM E. S. AND SAMUEL GURIN
Chemoreception in the shrimp, *Palaemonetes pugio:* comparative study of stimulatory substances in human serum 380

COCHRAN, ROGER C. AND FRANZ ENGELMANN
Environmental regulation of the annual reproductive season of *Strongylocentrotus purpuratus* (Stimpson) 393

DE VLAMING, VICTOR L.
Effects of photoperiod and temperature on gonadal activity in the cyprinid teleost, *Notemigonus crysoleucas* 402

HERNANDORENA, A.
Metabolic significance in nucleic acid metabolism and protein synthesis of dietary AMP requirement in *Artemia salina* (L.) 416

RIDDIFORD, LYNN M.
Juvenile hormone-induced delay of metamorphosis of the viscera of the Cecropia silkworm 429

SIMIONE, FRANK P., JR. AND DANIEL L. HOFFMAN
Some effects of eyestalk removal on the Y-organs of *Cancer irroratus* Say 440

STICKLE, WILLIAM B.
The reproductive physiology of the intertidal prosobranch *Thais lamellosa* (Gmelin). II. Seasonal changes in biochemical composition .. 448

WALKER, CHARLES WAYNE
Studies on the reproductive systems of sea-stars. II. The morphology and histology of the gonoduct of *Asterias vulgaris* 461

WHEELER, ALFRED P., PATRICIA L. BLACKWELDER AND KARL M. WILBUR
Shell growth in the scallop *Argopecten irradians.* I. Isotope incorporation with reference to diurnal growth 472

WOBBER, DON R.
Agonism in asteroids 483

Volume 148 Number 1

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

JOHN M. ANDERSON, Cornell University
JOHN B. BUCK, National Institutes of Health
JOHN O. CORLISS, University of Maryland
JOHN D. COSTLOW, Duke University
CATHERINE HENLEY, University of North Carolina
GEORGE O. MACKIE, University of Victoria
F. H. RUDDLE, Yale University
BERTA SCHARRER, Albert Einstein College of Medicine
HOWARD A. SCHNEIDERMAN, University of California, Irvine
GROVER C. STEPHENS, University of California, Irvine
CARROLL M. WILLIAMS, Harvard University
EDWARD O. WILSON, Harvard University

W. D. RUSSELL-HUNTER, Syracuse University
Managing Editor

FEBRUARY, 1975

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

6

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $7.00. Subscription per volume (three issues), $18.00, (this is $36.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between May 23 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

Copyright © 1975, by the Marine Biological Laboratory
Second-class-postage paid at Lancaster, Pa.

INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below· those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. *A condensed title* or running head of no more than 35 letters and spaces should be included.

Continued on Cover Three

Vol. 148, No. 1 February, 1975

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

COPPER UPTAKE AND EXCRETION BY *BUSYCON CANALICULATUM* L.

Reference: *Biol. Bull.*, **148**: 1–15. (February, 1975)

SUSAN B. BETZER[1] AND MICHAEL E. Q. PILSON

Graduate School of Oceanography, University of Rhode Island,
Kingston, Rhode Island 02881

Copper concentration in tissues of the channeled whelk, *Busycon canaliculatum* L., despite great variability, has been found to undergo a seasonal cycle, generally increasing in the early summer, when feeding begins, and decreasing in the fall and in the winter hibernation period (Betzer and Pilson, 1974). This metal is physiologically important, since marine gastropods require copper for the synthesis of the blood pigment, hemocyanin (Hcy). Thus it is of interest to know how they accumulate the trace substance, to what degree its concentration in their bodies is regulated, and to what environmental factors the seasonal copper cycle may be related. In the present study, uptake of copper into the whelk, sites of accumulation, and the possibility of copper regulation have been investigated in a series of uptake experiments using radioactively labeled copper as a tracer. Measurements were made of possible copper loss by excretion and by spawning.

MATERIALS AND METHODS

Fifty-four specimens of *Busycon canaliculatum* were obtained in Narragansett Bay, Rhode Island, and maintained in running bay water as described previously (Betzer and Pilson, 1974). Animal weights ranged from 100–200 g, in general.

Uptake experiments

Radioactive ^{64}Cu was prepared from copper shot dissolved in concentrated nitric acid and made to volume with deionized water to give a concentrated copper nitrate solution. Aliquots of this standard were neutron-activated, ordinarily for 6–8 hr, at a neutron flux of 4×10^{12} neutrons cm^{-2} sec^{-1} at the Rhode Island Nuclear Science Center.

---

[1] Present address: Department of Marine Science, University of South Florida, St. Petersburg, Florida 33701.

Copyright © 1975, by the Marine Biological Laboratory
Library of Congress Card No. A38–518

For experiments with ^{64}Cu uptake, a known amount of activated Cu standard was mixed into 3 liters of filtered (Whatman #42) freshly-collected, Narragansett Bay water in 4-liter polypropylene beakers. One whelk was placed in each beaker for the uptake period of 1–48 hr. At intervals during the uptake period, whelks were removed, put into square plastic refrigerator containers, and placed above a sodium-iodide crystal. Gamma emission from ^{64}Cu was counted, using a Tracerlab multichannel analyzer. All counts were corrected to time 0 using the decay constant for ^{64}Cu ($t_{\frac{1}{2}} = 12.8$ hours).

At the end of the uptake period, whole whelks were counted again and dissected. Whole organs (digestive gland, gut, kidney, gonad, osphradium, and gill), one-ml blood samples, and tissue samples (ordinarily foot muscle and mantle) were placed in preweighed plastic vials and counted in the well of the sodium-iodide crystal; the empty shells were also counted in refrigerator containers above the crystal. Each of the whole organs, blood and tissue samples was then weighed, dissolved, and analyzed for stable copper concentration by the spectrophotometric cuproine method or by atomic absorption spectroscopy, as described and reported previously (Betzer and Pilson, 1974).

An aliquot of activated copper standard equal to the amount added to each uptake beaker was counted in the crystal at the beginning of each experiment and the same aliquot was counted at the time of each subsequent count of whelks or tissues. The activity of the known amount of copper in the reference solution was used to convert all counts made in the well of the crystal to μg of labeled copper present in the substance counted.

A number of separations were carried out in trying to determine whether radioactive copper taken up in the blood had been incorporated in the hemocyanin molecule or was carried with a lower molecular weight fraction. The first technique used was ultracentrifugation (Ghiretti, 1966). A precounted labeled blood sample was diluted with 0.1 M KCl to fill the centrifuge tube and spun 1.5 hr at 360,000 *g* in a Beckman Ultracentrifuge. The content of radioisotope associated with the resulting pellet (containing the hemocyanin) and supernatant fluid was then determined. A second separation technique used was gel filtration. A 0.5 ml aliquot of each labeled precounted blood sample was applied to a column containing 30 ml of Sephadex® G-15 gel and eluted with 0.1 M KCl at a rate of 3.5–7 ml/hr. Fractions were collected automatically every 30 min and counted. The column was calibrated with a solution of unlabeled whelk blood and ^{64}Cu in seawater; fractions were collected, counted, and analyzed for protein by the Biuret method. The third technique for separating unbound copper from blood hemocyanin was that of Joselow and Dawson (1955), employing ion-exchange chromatography. Labeled precounted blood samples were applied to columns of Bio-Rex 40 (100–200 mesh) in the sodium form and passed through at about 5 ml/hr. The effluent was collected in 10-ml volumetric flasks and counted.

Excretion experiments

Sea water was passed through a Whatman #42 filter and then through a Chelex column to remove trace metals. Individual whelks were placed in polypropylene beakers containing 3 liters of this water and incubated up to 72 hr as was a blank beaker, with no whelk added. After removal of the whelks, the copper content of the water and any particulate matter was determined; the walls of the beaker were rinsed with 200 ml of 2 N nitric acid and the copper content determined.

The copper content of the seawater used was determined by passing the water through a column containing Chelex-100 ion-exchange resin in the hydrogen form, at a rate of about 5 ml/min (Riley and Taylor, 1968). The column was rinsed with 50 ml of deionized water and eluted with 200 ml of 2 N nitric acid, which was boiled down to a few ml in a Vycor® beaker covered with a Teflon® watch glass. The acid rinses of the beaker walls and the particulate matter were similarly boiled down. The solutions were transferred to 25-ml volumetric flasks, made up to volume with deionized water, and analyzed for copper by atomic absorption spectroscopy. Nitric acid blanks were run and the values subtracted from the copper concentrations in the samples.

RESULTS

Uptake of labeled dissolved copper by Busycon

In eight experiments a total of 34 whelks were exposed to concentrations of added copper of 3–6 μg/l (total copper concentration of 6–9 μg/l), comparable to the normal concentration of copper in Narragansett Bay water, 3 μg/l (D. Hallett, University of Rhode Island, personal communication). The amount of copper present in the incubation beakers was thus insignificant in comparison to the very large amount of total body copper in *Busycon* (averaging 7600 μg for a whelk of 100 grams—Betzer and Pilson, 1974). In one experiment, 4 whelks were exposed to high values of added copper, 106 μg/l. Incubation times for all the uptake experiments, between 1 and 48 hours, were limited by the short half-life of the isotope, 12.8 hours.

Sequence of uptake by the whole whelks. Because of the difference in geometry between whole whelks counted above the crystal and the 1 ml of reference solution counted in the well of the crystal, counts of whole whelks cannot be converted directly to μg of copper taken up by the animals. A conversion factor was obtained, however, using data from 4 experiments in which the activity of the whole uptake beakers, containing sea water and added labeled copper, was continued before and after incubation of the whelks. In each case a linear relationship was found between loss of activity in the beakers and gain in activity by the whelks which occupied them. Because the total amount of copper (μg) originally added to the beaker was known, the amount of copper taken up by a whelk could then be calculated from the slope of the graph. Since the activity (counts per minute) of the copper initially present in the beaker was proportional to the activity of the same amount of copper in the reference solution, a conversion factor was calculated from the counts per minute (cpm) in the whelk and the fraction of the copper in the reference solution represented by these counts. In four different uptake experiments the factor was calculated to be 4.20, 3.66, 4.26, and 3.79, giving an average of 4.0. This factor was applied in all uptake experiments to calculate, from counts of whole whelks above the crystal, the μg of labeled copper taken up

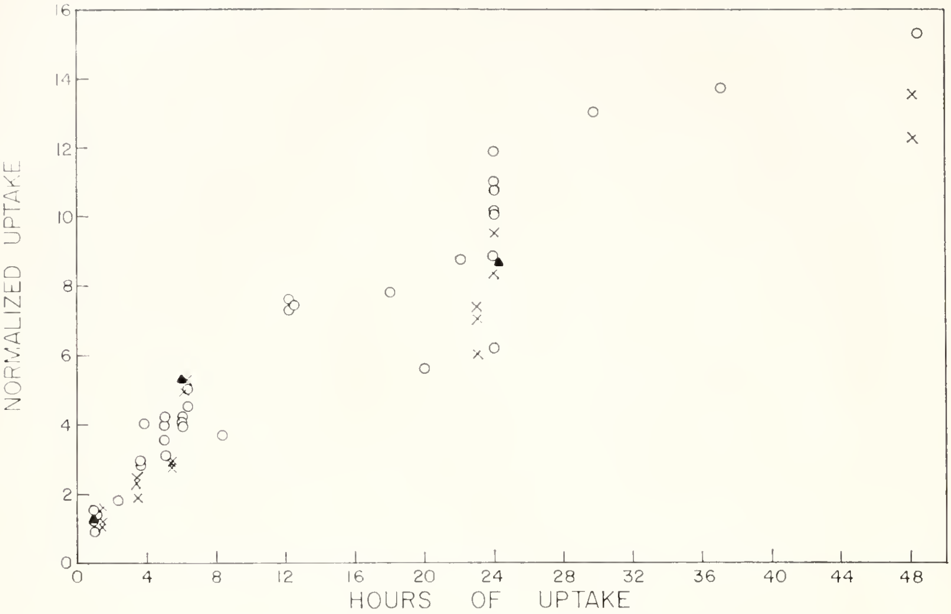


FIGURE 1. Uptake of dissolved, labeled Cu with time into whole whelks (weighing 100 g) from sea water. Points represent individual whelks exposed to different initial concentrations of added Cu in the beakers: diagonal crosses, 6 μg/l; open circles, 9 μg/l; closed triangles, 109 μg/l; temperature of experiments: 21° C.

by the whelks:

$$\frac{4.0\ (\text{cpm in whole whelk})\ (\text{total } \mu\text{g Cu in uptake beaker})}{\text{cpm of reference solution in crystal}}$$

$$= \mu\text{g Cu taken up into whelk.}$$

The validity of the conversion depends on the active copper being distributed in and on the body with approximately the same geometry from one whelk to the next. Whelks were always placed in the same orientation to the crystal during counting. The conversion factor was also applied to whelk shells counted above the crystal after dissection.

To allow comparisons among experiments in which different copper concentrations were used in the uptake medium, the concentrations of labeled copper taken up in both whole whelks and tissues have been normalized by dividing by the initial concentration of copper in the medium. This is referred to as the normalized uptake:

$$\text{Normalized uptake} = \frac{\mu\text{g Cu taken up per g of whelk}}{\text{initial } \mu\text{g Cu per ml of water in beaker}}.$$

Figure 1 shows the typical sequence of copper uptake into whole whelks from seven uptake experiments. The whelks showed a smooth, continuous increase in

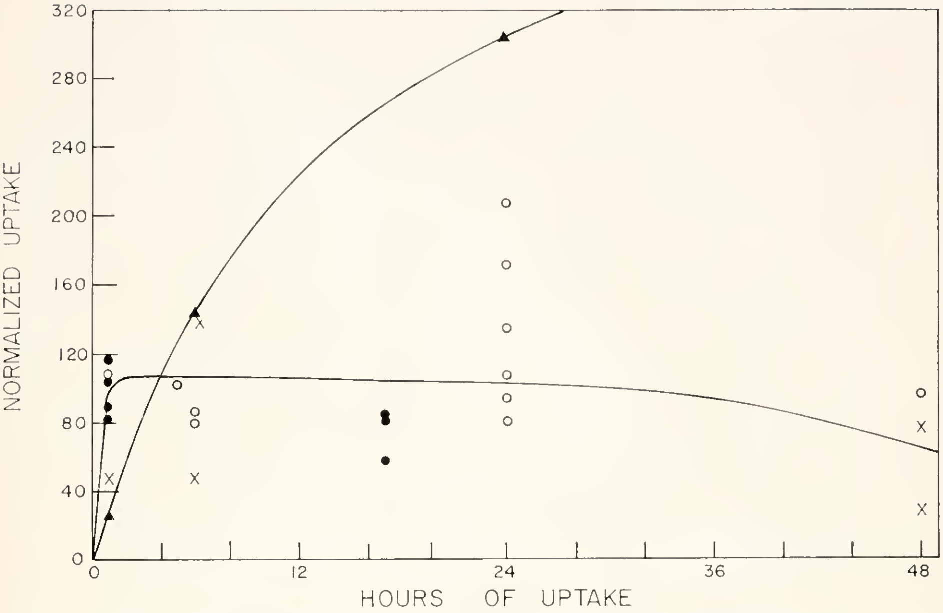


FIGURE 2. Uptake of dissolved, labeled Cu by gills with time. Points represent gills of individual whelks exposed to different initial Cu concentrations and temperatures of uptake: diagonal crosses, 6 μg/l and 17–21° C; open circles, 9 μg/l and 15–20° C; closed circles, 9 μg/l and 21–25° C; closed triangles, 109 μg/l and 15–20° C.

labeled copper, with the slope decreasing with time and leveling off toward 48 hr. This is probably due to a depletion of ^{64}Cu in the beaker; the three whelks incubated 48 hr had taken up 63%, 69%, and 78% of the total labeled copper originally present in the medium.

The effect of copper concentration on the rate of uptake into whole animals is apparent in Figure 1. Despite the individual variation, whelks exposed to 6, 9, and 109 μg Cu/l generally showed the same normalized uptake in the same length of time; *i.e.* uptake rate was directly proportional to the concentration of copper in the medium. Yager and Harry (1964) also found increased ^{64}Cu uptake in the freshwater snail, *Taphius glabratus,* when more copper was available.

Localization of copper taken up by whelks. Although the amount of labeled copper taken up into the individual tissues was small in comparison to their stable copper content, most tissues showed an accumulation of ^{64}Cu many times its initial concentration in the incubation medium. As in whole whelks, uptake rates in individual tissues and shells were directly proportional to copper concentration, except in the case of the gill and osphradium, as noted below.

Copper uptake onto the shell of *Busycon* followed the same general pattern as uptake by whole animals, with the normalized uptake increasing smoothly but with decreasing slope throughout the exposure period. This was not found by Yager and Harry (1964), whose measurements of ^{64}Cu uptake from 30 μg/l solutions by *Taphius glabratus* showed wide fluctuations and a decrease in copper

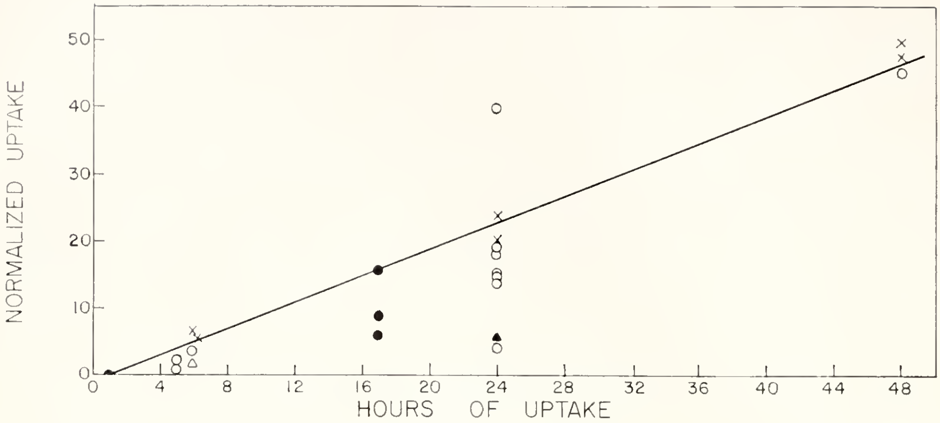


FIGURE 3. Uptake of labeled Cu by digestive gland with time. Points represent digestive glands of individual whelks exposed to different initial Cu concentrations and temperatures of uptake: diagonal crosses, 6 μg/l and 17–21° C; open circles, 9 μg/l and 15–20° C; closed circles, 9 μg/l and 21–25° C; closed triangles, 109 μg/l and 15–20° C. At 1 hr of uptake there are 6 points clustered at a normalized uptake of about 0.

concentration on the shell with time. In the present experiment the shell consistently accounted for about 30% of the total counts of the whole animal, and this copper was adsorbed on the surface. Counts of shells with a portable counter, after the whelk tissues had been dissected out, showed essentially all the activity on the outside. An empty shell incubated as a control in one experiment showed the same uptake rate as the shells of live whelks, suggesting that the inner nacreous layer adsorbed little copper in comparison to the hairy, organic periostracum.

Of the soft tissues, the gills (Fig. 2) and osphradium were the first to become strongly labeled. At low levels of copper in the medium, comparable to those of the environment, the gills had a normalized uptake of about 100 after 1 hr of incubation and may already have been saturated with copper. After this time the concentration in the gill showed little change, except for a possible decrease between 24 and 48 hr when the supply of labeled copper in the medium had become depleted. No difference in normalized uptake was distinguishable between the gills of whelks at 6 and 9 μg Cu/l; but gills of whelks at 109 μg/l showed a different pattern: not yet saturated at 1 hr, they continued to accumulate copper up to 24 hr, reaching the much higher normalized uptake of 300. The osphradium, a very small (0.1–0.2 g), gill-like structure adjacent to the gill in the mantle cavity, differed from the gill in showing a more gradual accumulation of copper with time. It also reached a much higher normalized uptake, leveling off at about 400 by 24 hr.

Despite individual variation, the kidney showed a fairly steady linear increase in labeled copper with time. The first of the internal organs to show significant uptake, it exhibited a concentration greater than or equal to that of the medium after 1 hr of incubation; by 24 hr it had a normalized uptake of 15–20. The gut and digestive gland (Fig. 3) showed little evidence of copper absorption at 1 hr;

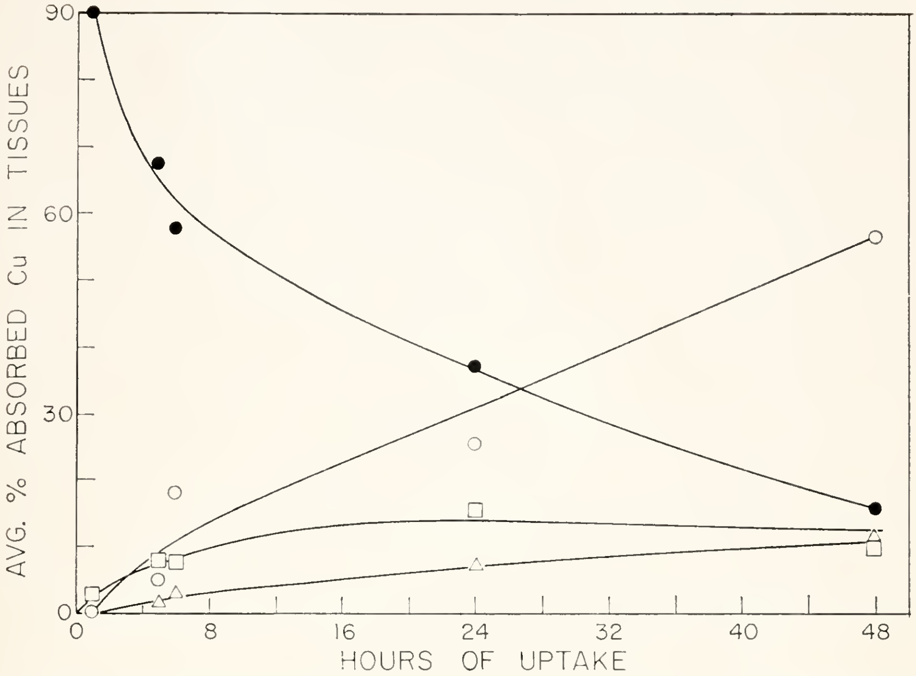


FIGURE 4. Sequence of uptake of Cu with time into the gill and tissues of the visceral mass of 18 whelks exposed to 6–9 μg Cu/l at a temperature of 15–20° C. The total Cu taken up into the gill, osphradium, digestive gland, gut, kidney, and gonad was summed, and the percentage contained in each tissue was calculated for each whelk. The average percentages for each uptake period were then plotted against the duration of uptake. Only the most quantitatively important tissues are shown: closed circles, average percentage in gill; squares, average percentage in kidney; open circles, average percentage in digestive gland; open triangles, average percentage in gut.

what little activity was found may have been due to the presence of blood. By 6 hr the normalized uptake was about 5 in both, and uptake continued at an approximately linear rate in both tissues for the rest of the incubation period, reaching a similar normalized uptake of about 45–60 by 48 hr, with no sign of leveling off.

The blood was labeled during the first hour, with a concentration of absorbed ^{64}Cu about equal to that initially present in the medium. More ^{64}Cu accumulated with time, but, in general, ^{64}Cu concentration in the blood after any exposure time (after 1 hr) was lower than in the kidney, digestive gland, or gut. Centrifuging blood samples to remove the cells was found not to affect the ^{64}Cu activity in the sample, indicating that the blood cells were not significant sites of copper accumulation.

Other tissues were routinely counted for copper uptake: foot muscle showed a low level of label, probably due mostly to the presence of blood; tissue exposed directly to sea water, such as the mantle and mucous gland, showed a slow increase in activity, probably due to surface adsorption as well as blood; the gonad was extremely variable but showed generally about the same concentration of active copper as the digestive gland and gut.

The metabolically active gill, osphradium, and visceral mass tissues together contained an average of 62% of the ^{64}Cu in *Busycon* tissues after 1 hour of incubation; they contained an average of 45% (range 30–50%) after 5–48 hours of incubation (as copper was accumulated in the blood and foot muscle). The sequence of changing distribution of absorbed copper among these metabolically active tissues is shown in Figure 4. Here, the total copper taken up into the gill, osphradium, digestive gland, gut, kidney, and gonad was summed, and the percentage found in the various tissues averaged for the different uptake periods. The total in the blood could not be included because the blood volume was not known. Because the speed of uptake in some tissues appeared to vary with temperature and copper concentration in the medium, data are presented here only for whelks exposed to 6–9 μg Cu/l, at temperatures of 15–20° C. At 1 hr of exposure, the gill had 90% of the total copper in these tissues, but although its total copper remained more or less constant (Fig. 2) it decreased in importance as copper in the other tissues rose, so that at 48 hr, the gill represented only about 15% of the total. The digestive gland had taken up negligible copper by 1 hr, but showed a steady increase in importance, so that it contained 50% of the copper at 48 hr. The kidney appeared to increase until about 24 hr, after which it leveled off or decreased slightly. The gut showed a slow increase over the 48-hr period, containing at the end of this time about 10% of the copper absorbed in these tissues. This pattern suggests that copper moves into the body at the gills and is transferred mainly to the digestive gland, although it is distributed to other tissues of the visceral mass as well.

This explanation is supported by the results of an "uptake and release" experiment. Three whelks which had been exposed to a concentration of 6 μg/l added labeled copper for 17 hr were counted whole and then transferred to Bay water with 6 μg/l added unlabeled copper for 24 hr before dissection and counting. Counts of radioactive copper in these whelks kept in unlabeled water for 24 hr after uptake were as high as at the end of the uptake period, showing that absorbed copper was not rapidly flushed out. Analysis of active copper in the water and particulate matter in the "release" beakers, after the removal of the whelks, showed that in all three whelks only about 7% of the label that had been taken up was released (an amount that was within the counting error for whole whelks). The absorbed copper was redistributed among the tissues during the 24 hr incubation in unlabeled water, in comparison with that of three other whelks dissected immediately after the 17 hr of uptake. Labeled copper in the gills decreased to less than half its concentration at 17 hr, while it increased strikingly in the kidney, gut, digestive gland, and gonad. There was no change in labeled copper levels in the blood. In terms of percentage of total labeled copper in gills, osphradium, and visceral mass tissue, the digestive gland increased from 15% to 51%.

Form of labeled copper in the blood and digestive gland. Ultracentrifugation of blood samples from 11 whelks from the uptake experiments yielded pellets (Hcy fraction) containing an average of 2/3 of the total label in the blood. Even when an excess of neutral, unlabeled copper was added to the sample before centrifugation, the bulk of the activity came down with the Hcy (and thus was not displaced). When labeled ionic copper was added to a sample of fresh whelk blood, 43% of the activity came down with the Hcy. This showed that the Hcy could bind or adsorb excess copper.

TABLE I.

Cu excretion by individual whelks

Excretion into water from which Cu had been removed

	Length of incubation	Total Cu released, µg	Cu excreted per 24 hr per 100 g
A. Feb. 1971 T = 25° C	24 hr	7.83	10.44
	48 hr	7.41	4.07
	72 hr	9.73	4.63
	72 hr	19.02	9.91
B. April 1971 T = 17–19° C	6¼ hr	4.00	18.6
	24 hr	3.16	2.75
	48 hr	9.60	4.00
C. July 1971 T = 21° C Aeration provided	6 hr	3.67	12.5
	24 hr	3.24	2.42
	48 hr	8.54	2.81
			$\bar{X}$ = 7.21 µg Cu/100 g per day

Excretion and uptake in water with added labeled Cu

	µg labeled Cu added	Length of incubation	Total Cu, µg, in beaker	Total minus blank	µg labeled Cu taken up by whelk*	Total Cu excreted	Cu excreted per 24 hr per 100 g
D. April 1971	17.6 µg	6 hr	17.8	−0.9	3.6	2.7	13.3
		24 hr	19.5	+0.7**	9.3	10.0	9.61
		48 hr	14.7	−4.0	12.1	8.1	3.51
		48 hr (no whelk) (blank)	18.7	—	—	—	—
E. July 1971	18 µg	6 hr	14.61	−4.85	9.06	4.21	6.86
		24 hr	17.70	−1.76	12.79	11.03	6.18
		48 hr	18.96	−0.50	14.09	13.59	5.96
		48 hr (no whelk) (blank)	19.46	—	—	—	—
							$\bar{X}$ = 7.57 µg Cu/100 g per day

* results from counts of radioactivity in uptake experiment.
** net excretion; all others show net uptake.

Labeled blood samples from 6 other whelks were passed through columns of Sephadex® gel; in each case the radioactive copper came through in the fraction containing the Hcy. Dissolved ionic copper and Hcy applied separately to Sephadex columns came through in different volume fractions, because of their difference in molecular weight; but when ^{64}Cu in sea water was mixed with a solution of unlabeled whelk blood, the copper activity came through in the same fraction with the Hcy. Thus the Sephadex gel filtration technique cannot distinguish the excess copper nonselectively bound by Hcy from copper incorporated in the active site of the Hcy molecule.

A similar ability to bind excess copper was found for lobster Hcy by Johnston and Barber (1969). They reconstituted lobster apohemocyanin with copper sulfate and with hepatopancreas supernatant and found that in the presence of excess copper, the apohemocyanin bound essentially all available copper—up to 6 times its original copper content.

In calibration experiments with BioRex 40 ion-exchange resin, *Busycon* blood passed through the column with its copper content unchanged, and labeled copper applied to the column in aqueous solution was quantitatively (99.8%) retained on the column. When labeled copper was mixed with *Busycon* blood, only a small amount of labeled copper (probably too small to have been detected with the counting techniques of Joselow and Dawson, 1955) passed through the column, 0.1–0.2 μg of a total of 3–10 μg copper applied.

In BioRex column separations of labeled blood samples from 11 whelks from the uptake experiments, a very small amount of labeled copper came through with the Hcy, 0–0.02 μg, or 0–37% of the initial labeled copper in the samples. There was no apparent correlation between the length of the uptake period and the amount of label that came through. Because this was less than 10% of the copper which had leaked through the columns in the calibration experiments, it is not known whether it represents incorporation in the Hcy, or "leakage" by the column. Since even with these small quantities of labeled copper, 63–100% of the labeled copper was removed from the blood sample by the column, it seems most likely that the labeled copper found in the blood in the uptake experiments was nonspecifically bound to the Hcy, not incorporated in the Hcy molecule.

A preliminary determination of subcellular distribution of copper in the digestive gland was made for two whelks incubated 24 hr with 6 μg/l added labeled copper. Differential centrifugation of the homogenized gland was followed by counts of each fraction for ^{64}Cu and analysis of each fraction for total copper. It was found that the newly taken up labeled copper was distributed differently than the total stable copper; 32–37% of the labeled copper remained in the supernatant after ultracentrifugation at 100,000 × g for 1 hour, while only 5–10% of the stable copper remained in this fraction, even the hemocyanin having sedimented. This indicated that a significant fraction of the newly absorbed copper was bound in some way to lower molecular weight substances, preventing the nonspecific binding that occurred between ionic copper and hemocyanin in previous centrifugation experiments and that probably occurs in transport of uptaken ionic copper by the blood.

Copper excretion by Busycon

A series of excretion or release experiments was carried out in order to determine whether the uptake of labeled copper by *Busycon* represented a net accumulation of copper or an exchange between the labeled copper in solution and the much larger pool of unlabeled copper in the whelk. Table I (A-C) presents the results of three experiments in which whelks were incubated in water previously stripped of trace metals. The net daily excretion per 100 g of soft tissue weight ranged from 2.42–18.6 μg copper, with a mean of 7.21 ± 5.42 ($1\ \sigma$). This value is not corrected for possible uptake by the whelks of copper they released; if this occurred, the total excretion would be greater. This could be the explanation for the higher rate of excretion by the whelks incubated for 6 hr in both Exps. B and C; by 6 hr only a comparatively small amount of copper had been released, and since rate of uptake is proportional to concentration, the competing effect of uptake on excretion was not seen until later.

Excretion was also measured in two of the uptake experiments described in the preceding section. After the removal of trace metals from the incubation medium, low concentrations of labeled copper were added; this made possible the simultaneous measurement of uptake and excretion. After the incubation period, the total copper in the water and particulate matter and on the walls of the beakers was determined and combined with the uptake data to give an estimate of total excretion. It has been assumed that copper taken up was not excreted, which seems reasonable, from the small amount of labeled copper lost in the uptake and release experiment. The results are presented in Table I (D, E). Daily copper excretion per 100 g of whelk ranged from 3.51–13.3 μg, averaging 7.57 ± 3.42 μg. This is roughly equivalent to the daily copper excretion when no copper was added and suggests that addition of copper to the medium did not increase the excretion rate. Since the copper excretion rate was about the same whether or not there was any initial copper in the water it seems likely that the copper which appeared was really excreted and not just desorbed from the shell surface. In the experiments where copper was added to the water the whelks maintained or increased their total body copper, instead of losing copper to the medium. Since the concentration of added copper in these experiments (6 μg/l) was not too different from that in the bay (3 μg/l), these results indicate that under ordinary conditions whelks probably remain more or less in balance, with dissolved copper taken in being equaled or only slightly exceeded by copper excreted. Where environmental copper concentrations are high, however, whelks may be expected to show a net accumulation from the medium, in addition to whatever copper is supplied in the diet.

Narcotizing with the gastropod relaxant and insecticide, Sevin®, according to the procedure of Carriker (1963), was found to have a pronounced effect on copper excretion by *Busycon*. In two uptake experiments, a whelk was narcotized with Sevin® before and during exposure to labeled copper. The whelks were dissected at 24 and 48 hr. Both whelks took up significantly less label than the other animals, and the shell of the 48-hr whelk had reduced label compared to the shell of normal whelks. In both narcotized whelks, uptake of labeled copper into internal tissues was negligible, and even in gills and osphradium, uptake was significantly reduced. These data were explained when the copper content of the beakers used for the 24 hr experiment was determined. The average of the other 5 beakers was 47 μg; the beaker which contained the narcotized whelk had 650 μg of copper. The Sevin® stock solution was analyzed; the 3.5 ml used in this experiment would have added only 0.245 μg copper to the beaker. The 650 μg of copper thus represented an excretion rate of 554 μg/day per 100 g tissue, about 75 times greater than the rates recorded in the excretion experiments. This massive release of unlabeled copper would tend to displace adsorbed labeled copper on the shell and body. The kidneys of the 2 narcotized whelks showed unusually low stable copper concentrations: that of the 48 hr whelk had 13 μg/g, and that of the 24 hr whelk had 20 μg/g, 40% and 30%, respectively, of the average concentrations in the kidneys of the other four whelks in each experiment, reported (as Feb. and Mar. averages) by Betzer and Pilson (1974).

Loss of copper through spawning. It was thought that the autumn reduction of the copper concentration in the blood and tissues of *Busycon* could be due to the fall spawning. In *Busycon,* eggs are fertilized internally and shed by the female enclosed in capsules connected as a long chain or egg string. Development from the egg to the juvenile stage takes place within the capsule. A measure of possible copper loss through spawning was made by determining the amount of copper in the contents of individual capsules obtained in late October, when the larvae had developed to the veliger stage. The larvae and associated albuminous material from ten capsules were ashed, dissolved in concentrated HCl, rinsed into volumetric flasks, and the copper content determined by the cuproine method (Diehl and Smith, 1958). The number of larvae per capsule ranged from 65–96, averaging 83. The copper present in the capsule contents ranged from 10–45 μg, averaging 23 μg. Thus, assuming 100 capsules/egg string (a reasonable average, from the data of Magalhaes, 1948), and no copper in the walls of the capsules (which were not analyzed), 2300 μg would be shed at spawning. This copper loss is much greater than the amount of change between pre- and post-spawning reproductive organs—less than 100 μg in a 120-g whelk (Betzer and Pilson, 1974), suggesting that perhaps copper from other tissues is mobilized and deposited in the eggs before spawning. Whether a similar loss of copper might occur in the male is not known.

Discussion

The fate of ^{64}Cu absorbed in *Busycon* agrees fairly well with that shown by studies of heavy metal uptake by a few other organisms (Bryan, 1964, 1968; Bryan and Ward, 1965; Hobden, 1969). In 6–42 hr of uptake of ^{64}Cu by the freshwater snail *Taphius glabratus* from solutions with a concentration of 31 μg Cu/l, Yager and Harry (1964) found the same magnitude of concentration in all tissues, except that in the liver it was 4–7 times higher. Townsley (1964) found that ^{64}Cu injected into the body of *Busycon* accumulated in the digestive gland.

From the pattern of uptake with time and the results of the "uptake and release" experiment, it seems that in *Busycon* the gills are probably the primary site of copper absorption from sea water, as they have been shown to be for other metals in other organisms (Bryan, 1968; Bryan and Ward, 1965). In whelks exposed to low, environmental concentrations of dissolved copper, the gills reached an "equilibrium" concentration by one hour, suggesting a balance between the rate of uptake by the gills and the rate of transport away to the other tissues. The higher normalized concentrations reached in the gills exposed to high copper concentration (109 μg/l) could be evidence that the mechanism of transport of copper from the gills into the body has become saturated at this concentration.

As discussed by Bryan (1968), it is not necessary to postulate active uptake of zinc by the gill of the lobster, *Homarus,* since most of the zinc in the blood and tissues is bound to a protein fraction; thus there is a concentration gradient of unbound zinc—higher in the medium, and lower in the gill. A similar argument may hold for *Busycon*; the results of the blood centrifugation, gel filtration, and column separations show that ionic copper is strongly bound by the blood Hcy of whelks, so that the concentration of ionic ^{64}Cu in the gill may actually be much lower than in the medium, regardless of the high concentration of total labeled copper.

The transfer of much of the absorbed copper to the digestive gland may represent a way of buffering the blood against high copper concentrations. Bryan (1964), in experiments with zinc uptake in *Homarus*, described the hepatopancreas as a "sponge which mops up excess zinc from the blood, and so, with the excretory organs, helps to keep the blood Zn level fairly normal" (page 556). We do not know whether the process of copper absorption by the digestive gland of *Busycon* is a regulated one, or a nonspecific uptake of the excess copper. The preliminary experiments with subcellular fractionation of the labeled digestive gland indicate that a substantial fraction of the copper newly taken up is held in association with low molecular weight substances—a different form than that in which it seems to be carried in the blood.

While the marked increase of copper in *Busycon* tissues during the early summer can be accounted for by the commencement of feeding (Betzer and Pilson, 1974), the mechanism for the drop in the fall and winter is not so easily explained. Typical copper loss from the blood (estimating blood volume as 30% as in *Buccinum*—Staaland, 1970) and digestive gland of an average whelk with tissue weight of 120 g can be calculated from the data of Betzer (1972) to yield a decrease of about 11,000 μg between summer and winter. Spawning was calculated to release perhaps about 2300 μg of copper. The rate of excretion, about 7 μg/day for a 100 g whelk, was balanced by the rate of uptake of dissolved copper in the incubation experiments, so that if excretion occurs at the same rate in the fall as in these experiments (conducted in the spring and summer), a large amount of copper could not be lost by this route. The striking rise in kidney copper concentration found by Betzer and Pilson (1974) in the late summer and early fall, when the other tissues are decreasing, however, indicates that perhaps increased copper excretion is occurring at this time of year. The copper content of three kidneys noticed for their unusual dark blue color at this season was more than five times higher than in typical winter animals. This could be evidence of a massive release of copper from apparently healthy fall whelks.

It appears that, in general, the copper content of *Busycon* is only very crudely regulated. Highly variable amounts of copper are undoubtedly consumed in the whelk diet, depending on the proportion of fish, shellfish, or other invertebrates consumed. Dissolved copper is taken up at apparently unregulated rates, depending on the concentration of the medium, so that it could be accumulated from polluted environments. Excretion of copper appears to be generally low and not related to the copper concentrations in the medium, although massive release of copper via the kidney in the fall could represent a coarse control on body copper concentration, along with release of copper at spawning. The highly variable tissue copper concentrations found in *Busycon* at all seasons of the year (Betzer and Pilson, 1974) attest to the small degree of regulation of this highly concentrated and (theoretically) metabolically important trace metal.

We wish to thank Richard Sisson, of the R. I. Dept. of Natural Resources, and Stanley Spink for providing specimens of *Busycon*. Stanley Cobb, Ross Wilcox, Kent Fanning, Patricia Kremer, and Jim Kremer helped in tending and feeding whelks and recording observations during long-term experiments. Robert Duce kindly made available his Perkin-Elmer 303 atomic absorption spectrophotometer; and George Tremblay, the Beckman Ultracentrifuge and other facilities of the Biochemistry Department of the University of Rhode Island. We are indebted to Stuart Kupferman for the suggestion of using radioactive labeling in tracing copper uptake, and to Donald Phelps, of the Environmental Protection Agency, and the staff of the R. I. Nuclear Science Center, particularly Mike Doyle and Frank DiMeglio, for use of the nuclear reactor and its laboratory facilities and for technical assistance. Peter Betzer assisted in many of the experiments, performed numerous copper analyses, and gave helpful suggestions throughout the preparation of the manuscript. This work was performed at the Graduate School of Oceanography of the University of Rhode Island, while one of us (S.B.B.) was supported by a National Science Foundation Graduate Fellowship.

SUMMARY

1. Pathways of copper into *Busycon canaliculatum* and sites of accumulation were investigated in uptake experiments using ^{64}Cu. Routes of possible copper loss were investigated in excretion experiments and by determination of copper content of egg capsules.

2. Uptake of dissolved ^{64}Cu by 38 whelks followed a smooth curve, slowing with time; about 2/3 of the available ^{64}Cu in 3 l of water was absorbed by 48 hr. The rate of uptake was proportional to the concentration of the medium.

3. Among the soft tissues, ^{64}Cu appeared first on the gills, which in 1 hr reached a normalized concentration 100 times that initially present in the medium, and in the blood and kidney (normalized concentration = 1 at 1 hr). By 6 hr of exposure, ^{64}Cu appeared in the gut and digestive gland (normalized concentration = 5).

4. The ^{64}Cu continued to accumulate in the digestive gland, so that by 48 hr, this tissue contained 50% of the total copper taken up by the gill and organs of the visceral mass. Transfer of absorbed copper to the digestive gland continued even when whelks were removed to unlabeled sea water for 24 hr.

5. Separations carried out on blood from whelks labeled with ^{64}Cu indicated that the absorbed copper in the blood was nonspecifically bound to hemocyanin.

6. Excretion rates for copper averaged 7 μg/24 hr per 100 g fresh tissue weight, and appeared unaffected by the copper concentration of the medium. Under normal environmental copper concentrations, rates of dissolved copper uptake and of copper excretion are probably about equal.

7. The average copper content of egg capsules was 23 μg/capsule. Spawning may be a significant route for copper loss, and an increase in copper excretion in autumn is also suggested as an explanation for a drop in tissue copper concentrations at this season.

LITERATURE CITED

BETZER, S. B., 1972. Copper metabolism, copper toxicity, and a review of the function of hemocyanin in *Busycon canaliculatum* L. *Ph.D. dissertation, University of Rhode Island*, 133 pp.

BETZER, S. B., AND M. E. Q. PILSON, 1974. The seasonal cycle of copper concentration in *Busycon canaliculatum* L. *Biol. Bull.*, **146**: 165–175.

BRYAN, G. W., 1964. Zinc regulation in the lobster *Homarus vulgaris*. I. Tissue zinc and copper concentraitons. *J. Mar. Biol. Ass. U.K.*, **44**: 549–563.

BRYAN, G. W., 1968. Concentrations of zinc and copper in the tissues of decapod crustaceans. *J. Mar. Biol. Ass. U.K.*, **48**: 303–321.

BRYAN, G. W., AND E. WARD, 1965. The absorption and loss of radioactive and non-radioactive manganese by the lobster, *Homarus vulgaris*. *J. Mar. Biol. Ass. U.K.*, **45**: 65–95.

CARRIKER, M., 1963. Some recent methods for narcotization, killing, fixation, and preservation of marine organisms. Pages 68–71 in H. D. Russell, Ed., *Notes on Methods for the Narcotization, Fixation, and Preservation of Marine Organisms.* Marine Biological Laboratory, Woods Hole, Massachusetts.

DIEHL, H., AND G. F. SMITH, 1958. *The Copper Reagents: Cuproine, Neocuproine, and Bathocuproine.* The G. F. Smith Chemical Company, Columbus, Ohio, 48 pp.

GHIRETTI, F., 1966. Molluscan hemocyanins. Pages 233–245 in K. M. Wilbur and C. M. Yonge, Eds., *Physiology of the Mollusca,* Vol. 2. Academic Press, New York.

HOBDEN, D. J., 1969. Iron metabolism in *Mytilus edulis*. II. Uptake and distribution of radioactive iron. *J. Mar. Biol. Ass. U.K.*, **49**: 661–668.

JOHNSTON, W., AND A. BARBER, 1969. Reconstitution of functional hemocyanin from apohemocyanin: the hepatopancreas as copper donor. *Comp. Biochem. Physiol.*, **28**: 1259–1273.

JOSELOW, M., AND C. R. DAWSON, 1955. Hemocyanin and radioactive copper. *Science,* **121**: 300–303.

MAGALHAES, H., 1948. An ecological study of snails of the genus *Busycon* at Beaufort, N.C. *Ecol. Monog.*, **18**: 377–409.

RILEY, J. P., AND D. TAYLOR, 1968. Chelating resins for the concentration of trace elements from seawater and their analytical use in conjunction with atomic absorption spectrophotometry. *Analytica Chim. Acta,* **40**: 479–485.

STAALAND, H., 1970. Volume regulation in the common whelk, *Buccinum undatum* L. *Comp. Biochem. Physiol.*, **34**: 355–365.

TOWNSLEY, S. J., 1954. Studies on copper in mollusks, with particular reference to *Busycon canaliculatum* Linnaeus. *Ph.D. dissertation, Yale University,* 126 pp.

YAGER, C. M., AND H. W. HARRY, 1964. The uptake of radioactive zinc, cadmium, and copper by the freshwater snail, *Taphius glabratus*. *Malacologia,* **1**: 339–353.

Reference: *Biol. Bull.*, **148**: 16–25. (February, 1975)

COPPER TOXICITY IN *BUSYCON CANALICULATUM* L.

SUSAN B. BETZER[1] AND PAUL P. YEVICH

Graduate School of Oceanography, University of Rhode Island,
Kingston, Rhode Island 02881 and
National Marine Water Quality Laboratory,
West Kingston, Rhode Island 02892

Copper is among the most toxic of the heavy metals to most organisms (Bowen, 1966; Bryan, 1971), and it may be introduced in significant amounts into the coastal marine environment from industrial sources. It thus seemed desirable to examine the tolerance for copper of a commercial organism commonly found in estuarine and coastal regions, the channeled whelk, *Busycon canaliculatum*. The effects of high copper concentrations on marine prosobranch gastropods such as *Busycon* are of particular interest, because these snails (like several other groups of molluscs and arthropods) normally accumulate and store copper and use it in the synthesis of the blood pigment, hemocyanin. Studies of the copper metabolism of *Busycon* under normal environmental conditions have been described elsewhere (Betzer, 1972; Betzer and Pilson, 1974). This paper presents a series of experiments carried out to determine the toxic concentration of copper for *Busycon* and to investigate the effects of high copper concentrations by determination of tissue copper concentrations, by tracing uptake with ^{64}Cu, and by histological examination.

Materials and Methods

Specimens of *Busycon canaliculatum* collected in pots from the Wickford-Fox Island region of Narragansett Bay, Rhode Island, were placed, 2 or 3 whelks per tank, in 8-liter all-glass aquaria. The tanks were filled with bay water to which various volumes of a cupric chloride stock solution had been added, so that the final concentration of added copper was between 0 and 1000 μg/l. The tanks were covered, aerated, and incubated in a wet table of running bay water to maintain the same temperature as in the natural environment (from 13–15° C in early June to 20–22° C in August). Every three to four days the water was changed and fresh copper stock was added. Animals that died were removed for copper determination. The whelks were not fed, unless otherwise noted.

For tissue copper determinations, exposed and control whelks were bled and dissected. Blood and tissue samples (gut, digestive gland, kidney, gonad, gills, osphradium, and foot muscle) were digested with aliquots of a solution prepared by mixing 100 ml of concentrated perchloric acid and 400 ml of concentrated nitric acid. The samples were analyzed for copper by the spectrophotometric cuproine method (b) of Diehl and Smith (1958) or by atomic absorption spectroscopy, as described elsewhere (Betzer and Pilson, 1974).

---

[1] Present address: Department of Marine Science, University of South Florida, St. Petersburg, Florida 33701.

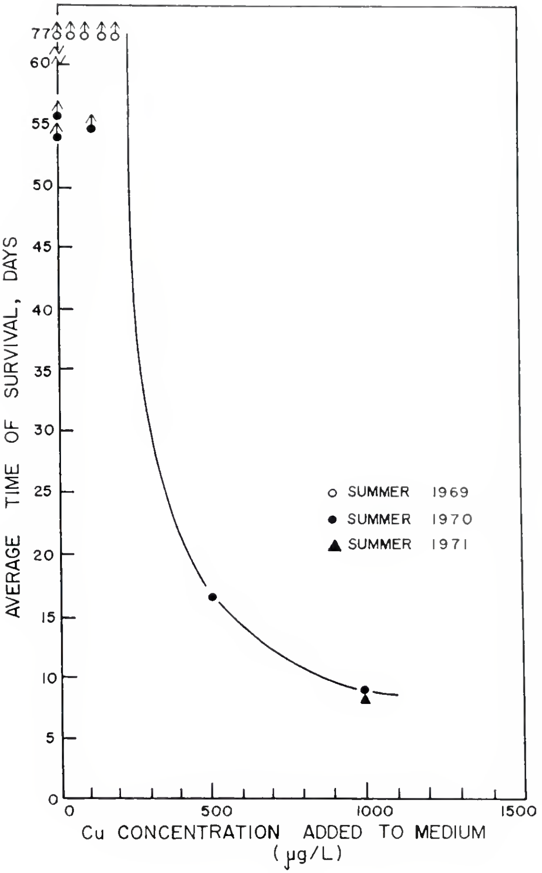


FIGURE 1. Average time of survival for whelks exposed to various copper concentrations; open circles, average time of survival for 6 whelks exposed to each concentration, summer 1969; closed circles, average time of survival for 4 whelks exposed to each concentration, summer 1970; closed triangles, average time of survival for 8 whelks exposed to 1000 μg/l added copper, summer 1971. Arrow indicates that the whelks were still alive when the experiment was terminated.

The sequence of uptake of copper into the tissues from both toxic (470 μg/l) and nontoxic (6 μg/l) solutions was followed using radioactively labeled ^{64}Cu added to bay water, from which trace metals had previously been removed by passage through a Chelex column (Riley and Taylor, 1968). Individual whelks were held in polypropylene beakers containing 3 liters of labeled solution for 6, 24, and 48 hr. After incubation the whelks were counted whole above a sodium-iodide crystal for gamma emission due to copper, using a multichannel analyzer. The whelks were dissected, and blood and tissue samples were also counted to determine ^{64}Cu uptake as described previously (Betzer, 1972).

Tissues of control whelks and whelks exposed to toxic copper concentrations (1000 μg/l added copper) for various periods of time were examined for evidence

TABLE I.

Copper concentrations (μg/g fresh weight) of gills and osphradia of whelks exposed to various Cu concentrations

	# of whelks	Added Cu μg/l	Days exposure	Gills		Osphradium	
				$\bar{X}$ ± S.D.	range	$\bar{X}$ ± S.D.	range
July–August 1970	4	0	54	35± 6	29– 41	16± 0.7	15– 16
	4	100	54	43± 10	32– 54	25± 4	23– 30
	4	500	$\bar{X}$ = 16 (all died)	316±158	215–551	96±40	64–151
	4	1000	$\bar{X}$ = 9 (all died)	231± 73	128–298	78±31	38–108
August 1971	3	0	0	77± 11	66– 89	45±10	39–56
	4	0	9	89± 22	58–107	40±10	27–52
	3	1000	4*	112± 16	96–128	60±22	35– 73
	4	1000	7 (one died)	188± 90	55–246	112±18	94–134
	4	1000	9 (three died, one dying)	185± 40	129–216	116±18	92–131

* transferred to an aquarium without added copper for a few hours before dissection.

of histopathology. Tissues were normally fixed in a modified Zenker-formol fixative, routinely processed, cut to a thickness of 6 μ, and stained with hematoxylin and eosin. For a histochemical study of copper deposition, the tissues were prepared according to the rubeanic acid method of Uzman (1956).

RESULTS

Copper tolerance of Busycon

In a preliminary experiment in June, 1969, a total of 30 whelks were incubated in 10 tanks containing 0, 50, 100, 150, and 200 μg/l added copper, with 2 tanks at each concentration. These concentrations were chosen because Marks (1938) had found that in similar experiments the limits of Cu tolerance for all gastropods tested were 100–200 μg/l. Four times during the 77-day course of the experiment, blood samples were removed from each animal in connection with another study. A small quahog was added to each tank as food on days 62, 72, and 74. During the course of the study, the mortality in the high copper tanks (150 and 200 μg Cu/l) was 50%, the same as in the control tanks (no added copper). Thus it seemed that the high ionic copper concentration of the medium was probably not responsible for the whelk deaths, but that death was more likely due to infection or injury during bleeding.

In a second experiment begun in July, 1970, 16 whelks were incubated, 2 per tank, in a broader range of added copper concentrations: 0, 100, 500, and 1000 μg/l, with 2 tanks at each concentration. After 54 days the experiment was terminated, and all the whelks were dissected for tissue copper determinations. All whelks at 0 and 100 μg/l survived the entire experiment and could often be seen actively crawling about the tank, adhering to the glass walls. During exposure to the higher copper concentrations, however, whelks frequently remained withdrawn into their shells, emitting large quantities of mucus. Figure 1 presents

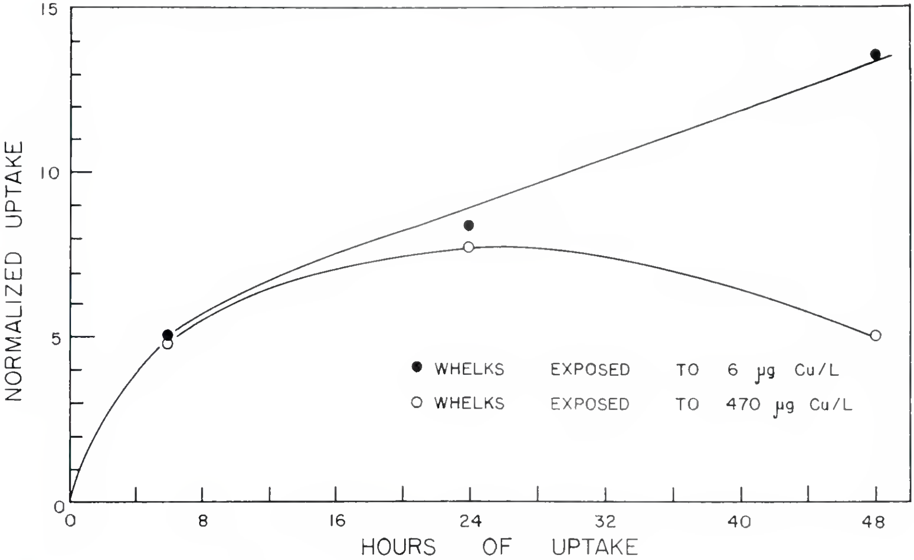


FIGURE 2. Uptake of ^{64}Cu by whole whelks, expressed as:

$$\text{Normalized uptake} = \frac{\mu\text{g Cu taken up per g of whelk}}{\mu\text{g/ml Cu initially present in medium}};$$

closed circles, whelks exposed to 6 µg/l added copper; open circles, whelks exposed to 470 µg/l added copper.

the average time of survival for whelks at various copper concentrations in this experiment, as well as in the preliminary experiment of the previous summer, and for whelks exposed to 1000 µg/l copper in August, 1971. In 1970, at 500 µg/l, the whelks survived an average of 16.5 days (range = 11–27 days); at 1000 µg/l, an average of 9 days (range = 7–10.5 days). In 1971, whelks exposed to 1000 µg/l added copper, and removed at intervals for chemical and histological copper determinations, survived 8 days (range = 7–9 days). Thus for these periods of exposure (54 and 77 days) the tolerance limit of *Busycon* for copper is between 200 and 500 µg/l.

Tissue copper concentrations of whelks exposed to various copper concentrations

Copper concentrations were determined for the 16 whelks exposed to various concentrations in the 1970 toxicity study and for 18 whelks exposed to 0 and 1000 µg/l added copper in August, 1971. In the 1971 experiment, 11 whelks exposed to 1000 µg Cu/l were dissected after incubation periods of 4 days (3 whelks), 7 days (4 whelks), and 9 days (4 whelks); control whelks were dissected after incubation periods of 0 days (3 whelks) and 9 days (4 whelks). In both experiments, whelks exposed to toxic copper concentrations showed marked increases in the amount of copper on the osphradium and, particularly, the gills (Table I). In 1970 there was no significant increase in whelks exposed to a high but sublethal copper concentration (100 µg/l), but there was a striking increase in the whelks

TABLE II

Concentration of uptaken ^{64}Cu and normalized uptake in tissues of whelks exposed to 6 and 470 μg Cu/l

Tissue	Medium	μg Cu taken up/g fresh weight			Normalized uptake*		
		6 hr	24 hr	48 hr	6 hr	24 hr	48 hr
Gills	low Cu:	0.29	0.49	0.18	48	82	30
	high Cu:	31	30	37	66	64	79
Osphradium	low Cu:	0.40	1.1	0.36	67	187	61
	high Cu:	37	27	32	79	58	68
Kidney	low Cu:	0.056	0.13	0.11	9.3	22	18
	high Cu:	1.3	2.0	2.7	2.8	4.4	5.9
Gut	low Cu:	0.041	0.22	0.37	6.8	37	61
	high Cu:	0.88	1.3	2.2	1.9	2.8	4.8
Digestive Gland	low Cu:	0.04	0.14	0.30	6.7	24	49
	high Cu:	0.39	0.43	0.72	0.84	0.93	1.5
Blood	low Cu:	0.017	0.029	0.037	2.9	4.9	6.1
	high Cu:	0.61	0.33	0.35	1.3	0.71	0.75

$$* \text{ Normalized uptake} = \frac{\mu g \text{ Cu taken up per gram whelk}}{\text{initial Cu concentration of medium, } \mu g/ml}.$$

that died from copper toxicity at 500 and 1000 μg/l. In 1971, concentrations were increased after 4 days of exposure to 1000 μg Cu/l and more than doubled after 7 and 9 days. The differences in concentration between the control groups of the 2 experiments were probably due to differences in time of capture (July of 1970 and August of 1971) and feeding histories (Betzer and Pilson, 1974).

Exposure of whelks to high copper concentrations had no apparent effect on the copper concentrations in the blood, kidney, or muscle, which showed quite similar concentrations in all groups within an experiment. In the case of the gut, gonad, and particularly the digestive gland, which has a copper-storing function, there was tremendous individual variation in the copper content within experimental groups—as much as 10-fold. This variation is characteristic of *Busycon* (Betzer and Pilson, 1974) but it obscured possible copper accumulation resulting from exposure to high copper concentrations.

^{64}*Cu uptake by* Busycon *from a toxic concentration*

Previous work has shown that *Busycon* can take up labeled copper from nontoxic seawater solutions containing 6–100 μg/l (Betzer, 1972). In the present experiment, 3 whelks were also exposed to toxic concentrations (470 μg/l) which caused them to withdraw into the shell and emit mucus even within the short periods of incubation used (6, 24, and 48 hr). Figure 2 presents uptake by the whole whelks, expressed as the ratio of μg of copper taken up per gram of whelk to the initial copper concentration of the incubation medium ("normalized up-

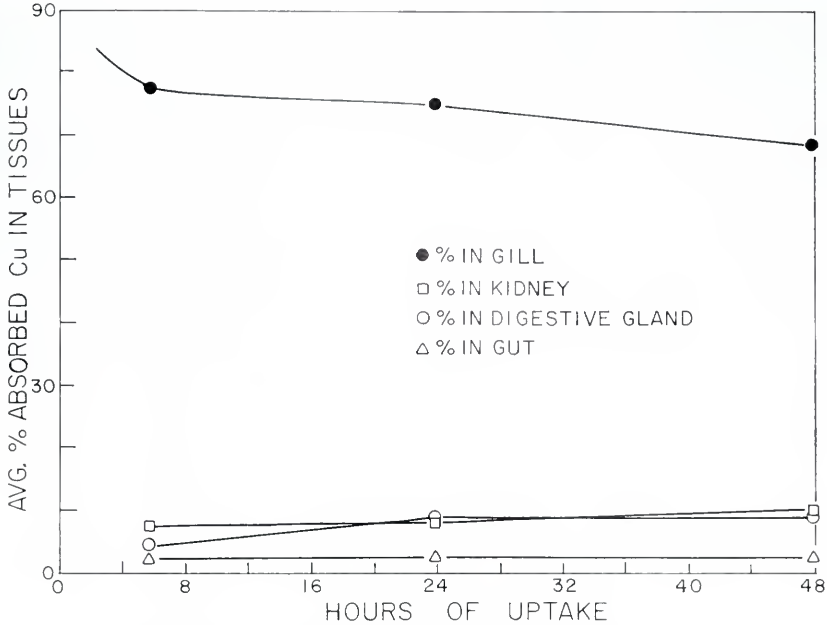
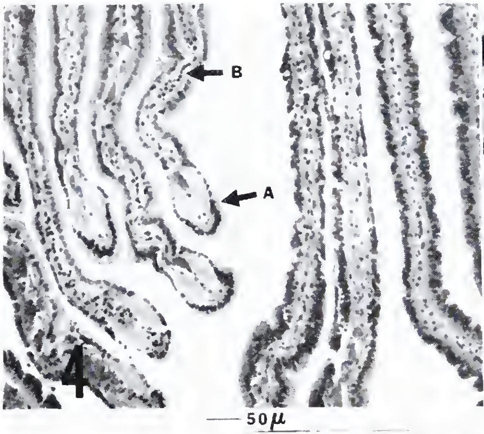


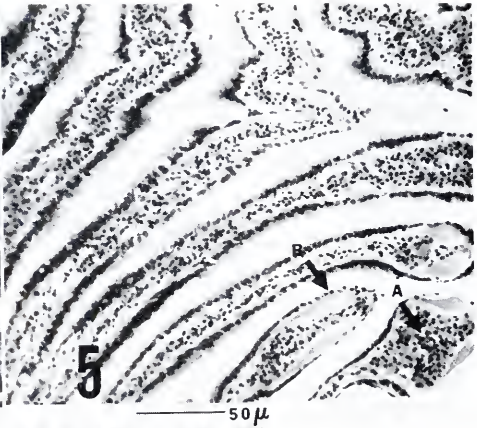
FIGURE 3. Sequence of copper uptake into gill and most important visceral tissues with time from a concentration of 470 μg/l. The total amount of copper taken up into the gill, osphradium, digestive gland, gut, kidney, and gonad was summed and the percentage found in each tissue was plotted for each uptake period; closed circles, percentage in gill; squares, percentage in kidney; open circles, percentage in digestive gland; open triangles, percentage in gut.

take"); results of uptake by 3 whelks exposed to low, environmental copper concentrations are plotted for comparison. Until 24 hr, whelks exposed to both copper concentrations showed increasing concentrations of labeled copper, but there was a decrease by 48 hr in the whelk exposed to toxic copper. In the low-copper whelks in this experiment, the shell accounted for an average of 44% of the total copper taken up; but the shell of the whelks at the toxic concentration accounted for an average of 78% of the copper taken up. Thus, at the toxic concentration, a smaller proportion of the uptaken copper was in or on the soft tissues.

Copper uptake by individual soft tissues is presented in Table II. Uptake of copper by the gills and osphradia of whelks in the medium with toxic copper was on the order of 50–80 times the initial concentration of the medium, similar to that of whelks exposed to low concentrations of added copper. In the whelks exposed to low copper concentrations, uptake by the gill and osphradium was high at 6 hr, increased until 24 hr, and then decreased again by 48 hr, as ^{64}Cu in the medium was depleted. The normalized uptake of gills and osphradia at the toxic concentrations remained about the same from 6 hr through 48 hr.

The internal tissues and organs, on the other hand, generally increased in concentration of absorbed copper in both groups. The quantities of labeled copper taken up were larger in the whelks exposed to toxic concentrations; but in both





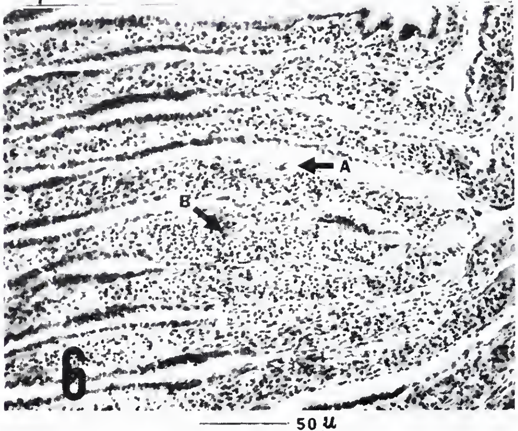


FIGURE 4. Gills of a control whelk, showing leaflet (Arrow A) structure of the organ. The inner area (Arrow B) of the leaflet shows normal blood lacunae; Zenker's, H & E.

FIGURE 5. Gills of a whelk exposed 3 days to toxic copper, showing dilation of blood sinuses filled with blood cells (Arrow A). The mucosa at the tips of the leaflets shows some regions of necrosis and sloughing (Arrow B); Zenker's, H & E.

FIGURE 6. Gills of a whelk exposed 7 days to toxic copper, showing extensive necrosis of the swollen gill leaflets (Arrow A), which lack epithelium, and amebocytic infiltration of the whole area (Arrow B); Zenker's, H & E.

groups, the amount taken up was small in comparison to the amount present in the tissues. The uptake normalized for initial concentration of the medium was 2–33 times lower, however, in whelks exposed to 470 μg/l than in those exposed to 6 μg/l. After 48 hr, the kidney, gut, digestive gland, and blood of whelks in the toxic medium had not yet reached the normalized uptake shown at 6 hr by whelks taking up copper from low concentrations. Thus the rate of uptake of labeled copper into these internal tissues from the toxic medium was not proportional to the rate of accumulation on the gills.

To follow the sequence of copper uptake into important tissues of the mantle cavity and visceral mass, the total labeled copper taken up into the gills, osphradium, digestive gland, gut, kidney, and gonad can be summed, and the percentage found in the various tissues computed for each uptake period. (The blood is omitted because the blood volume was not known.) In whelks exposed to 6 μg/l labeled copper, the gill had 90% of the uptaken copper after 1 hr; but as copper entered the body, the gill decreased in importance so that by 48 hr it contained only 15% of the total. Meanwhile, the digestive gland showed a steady increase in accumulation so that at 48 hr it contained 50% of the uptaken copper. This is the typical pattern for *Busycon* at normal environmental copper concentrations (Betzer and Pilson, 1975). Figure 3 is a graph of uptake into gills and tissues of the visceral mass for the 3 whelks incubated in 470 μg/l added copper. Here, the gill also had a high percentage of the total uptaken copper early in the incubation, but it maintained its importance throughout the 48-hr period. The digestive gland did not show the dramatic rise in copper accumulation that occurs in normal copper concentrations. Thus these data also suggest that proportionately less copper is being taken into the body from the gill.

Histopathologic findings for whelks exposed to toxic copper

The tissues of 3 control whelks and whelks exposed to 1000 μg/l added copper for 3 days (4 whelks), 4 days (2 whelks), 5 days (4 whelks), and 7 days (3 whelks) were examined for evidence of histopathology. Animals examined after 3 days of exposure to toxic levels of copper (Figure 5) showed dilated efferent blood sinuses and blood lacunae in the leaflets of the gill in comparison with control whelks (Figure 4). The blood sinuses were also dilated in the leaflets of the osphradium. The dilated areas were filled with a pink-staining material and showed a tremendous increase in amebocytes. This may be considered a type of inflammatory response in *Busycon*. There were also noted, in a few leaflets of the gills, focal areas of necrosis and sloughing of epithelial cells.

With increased exposure time (4, 5, and 7 days) there was a progressive increase in the swelling of the leaflets and in the inflammatory response, and in the necrosis and sloughing of the epithelium of the osphradium and gills (Fig. 6). The leaflets were in some cases completely denuded of epithelium, leading to necrosis of the various structures of the septum. At times the tips of the denuded septum ballooned out.

Microscopic examination of the heart, kidney, digestive gland, salivary glands, gut, foot, radula, reproductive tract, and mantle did not show any changes which could be attributed to the action of copper. Histochemical study of all tissues for copper deposition according to the method of Uzman (1956) did not show any differences between exposed and control animals. This does not seem to be a useful technique for studying copper deposition in *Busycon*.

DISCUSSION

The limits of copper tolerance for *Busycon*, between 200 and 500 μg/l, are high in comparison to the concentrations encountered in the natural environment; unpolluted Narragansett Bay water has a concentration of 3 μg/l (D. Hallett, University of Rhode Island, Graduate School of Oceanography, personal communication). Other mollusks have shown a greater sensitivity to copper than *Busycon*.

Marks (1938) found in experiments similar to those described here that for 10 species of Pacific coast mollusks the upper limit of copper tolerance was 100–200 μg Cu/l; except for one species of clam, none survived more than 18 days at 200 μg/l added copper. This was the same toxicity threshold found for *Mytilus edulis* (Scott and Major, 1972). Shuster and Pringle (1968) exposed quahogs (*Mercenaria mercenaria*) to copper concentrations of 25 and 50 μg/l and found 63% and 78% mortality by the 15th week, although oysters showed only 10% and 15% mortality after 20 weeks at the same concentration. Harry and Aldrich (1963) found that the freshwater pulmonate snail, *Taphius glabratus*, showed distress after only 24 hr of exposure to concentrations of 50–100 μg/l added copper.

As discussed by Bryan (1971), temporary storage of a metal in a particular tissue is a method of removing it from the rest of the body, and consequently reducing its toxic effects. Such accumulation of ^{64}Cu by the digestive gland is seen in *Busycon* exposed to labeled solutions of low (6 μg/l), non-toxic concentrations; and this, followed by later excretion of copper, may be the mechanism by which whelks resist higher copper concentrations.

Yager and Harry (1964) exposed *Taphius glabratus* to concentrations of labeled copper which allowed normal behavior and to concentrations which caused distress; they found that the livers of distressed snails contained less absorbed labeled copper than those of normal snails, and concluded that distress was somehow produced by disruption of membrane permeability, although the site of copper absorption was not identified. In *Busycon*, experiments using ^{64}Cu show that under normal concentrations, copper is taken up and transferred to the internal tissues; at toxic copper concentrations, where only the gills (and osphradium) show tissue damage, the rate of transfer into the internal tissues, particularly the digestive gland, is sharply decreased. These results indicate that the gills are the primary site of entrance of dissolved Cu into the whelk body, rather than other possible routes such as through the gut or the general body surface.

The histopathologic findings in *Busycon* are much the same as those seen in previous studies by Yevich (unpublished) after the exposure of the fish, *Fundulus heteroclitus*, and the quahog, *Mercenaria mercenaria*, to toxic concentrations of copper. There, too, the main organ involved was the gill, in which there was necrosis and sloughing of the epithelium, and, in *Fundulus*, a ballooning of the tips of the gill filaments. In *Busycon*, the clear evidence of structural damage to the gill at toxic concentrations suggests that there may be interference with respiration, as well as with normal copper transport mechanisms into the body.

We thank the staff of the Rhode Island Nuclear Science Center for technical assistance and use of their equipment, Peter Betzer for help in carrying out the experiments, and Michael Pilson and Norman Blake for help in preparing the manuscript.

SUMMARY

1. The effects of high concentrations of copper in seawater upon *Busycon canaliculatum* were followed histologically, by determination of tissue Cu concentrations, and by tracing uptake with radioactively labeled copper (^{64}Cu).

2. Whelks showed a high resistance to ionic Cu, with a tolerance limit between 200–500 μg/l at normal habitat temperatures for the exposure periods used (54–77 days).

3. At lethal concentrations, Cu was accumulated at the gill and osphradium; and these tissues also showed progressive histopathologic change, consisting of swelling of the gill filaments, amebocytic infiltration of the connective tissue, and necrosis and sloughing of the mucosa.

LITERATURE CITED

Betzer, S. B., 1972. Copper metabolism, copper toxicity, and a review of the function of hemocyanin in *Busycon canaliculatum* L. *Ph.D. dissertation, University of Rhode Island,* 133 pp.

Betzer, S. B., and M. E. Q. Pilson, 1974. The seasonal cycle of copper concentration in *Busycon canaliculatum* L. *Biol. Bull.,* **146**: 165–175.

Betzer, S. B., and M. E. Q. Pilson, 1975. Copper uptake and excretion by *Busycon canaliculatum* L. *Biol. Bull.,* **148**: 1–15.

Bowen, H. J. M., 1966. *Trace Elements in Biochemistry.* Academic Press, New York, 241 pp.

Bryan, G. W., 1971. The effects of heavy metals (other than mercury) on marine and estuarine organisms. *Proc. Roy. Soc. London Series B,* **177**: 389–410.

Diehl, H., and G. F. Smith, 1958. *The Copper Reagents: Cuproine, Neocuproine, and Bathocuproine.* The G. F. Smith Chemical Company, Columbus, Ohio, 48 pp.

Harry, H. W., and D. Aldrich, 1963. The distress syndrome in *Taphius glabratus* (Say) as a reaction to toxic concentrations of inorganic ions. *Malacologia,* **1**: 283–287.

Marks, G. W., 1938. The copper content and copper tolerance of some species of mollusks of the southern California coast. *Biol. Bull.,* **75**: 224–237.

Riley, J. P., and D. Taylor, 1968. Chelating resins for the concentration of trace elements from seawater and their analytical use in conjunction with atomic absorption spectrophotometry. *Analytica Chim. Acta,* **40**: 479–485.

Shuster, C. N., and B. H. Pringle, 1968. Effects of trace metals on estuarine mollusks. Pages 285–304 in *Proceedings, First Mid-Atlantic Industrial Waste Conference,* University of Delaware, CE-5.

Scott, D. M., and C. W. Major, 1972. The effect of copper (II) on survival, respiration, and heart rate in the common blue mussel, *Mytilus edulis. Biol. Bull.,* **143**: 679–688.

Uzman, L. L., 1956. The histochemical localization of copper with rubeanic acid. *Lab. Invest.,* **5**: 299–305.

Yager, C. M., and H. W. Harry, 1964. The uptake of radioactive zinc, cadmium, and copper by the freshwater snail, *Taphius glabratus. Malacologia,* **1**: 339–353.

Reference: *Biol. Bull.*, **148**: 26–34. (February, 1975)

THE ANOMALOUS INFLUENCE OF SALINITY ON TEMPERATURE TOLERANCES OF SUMMER AND WINTER POPULATIONS OF THE COPEPOD *EURYTEMORA AFFINIS*

BRIAN P. BRADLEY

Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, Maryland 21228

One of the more important variables in the estuaries and other environments is temperature. In these environments, survival of organisms, especially the more passive forms, depends on their tolerance to daily and seasonal fluctuations in temperature.

Temperature tolerances are in turn modified by other variables such as salinity and dissolved oxygen. These three variables are seasonally related in the estuary. Dissolved oxygen is physically dependent on temperature and to some extent salinity.

Previous work on temperature effects on marine invertebrates is reviewed by Kinne (1963, 1964, 1967, 1970). General treatments appear in Remane and Shlieper (1971) and in Vernberg and Vernberg (1972). These works also include discussions of the modifying effects of other variables. The classic paper on interacting variables is by McLeese (1956), working with the lobster *Homarus americanus*, who found that temperature of acclimation, salinity and oxygen tension all influenced the upper lethal temperature.

The present paper reports on experiments which were designed to find a suitable assay for thermal tolerance in the calanoid copepod *Eurytemora affinis* (Poppe), to investigate the effect of salinity on temperature tolerance and to compare the tolerances of populations of *E. affinis* collected at different seasons.

METHODS

The cultures of *E. affinis* were raised in sterilized bay water using the method of Heinle (1969b). Mature males were used in all the experiments, although no sex differences in thermal tolerance were detected in preliminary studies.

Elevated temperatures were obtained using a thermostatically controlled heating-stirring unit in an aquarium in which shell vials containing the test animals were placed. Water temperature was carefully monitored and no temperature gradients were detected in the aquarium. Time lags in temperature in the vials were 34–90 seconds, depending on the external temperature. Low temperatures were obtained using a cold room (2° C) and the heating unit used if necessary.

High temperature tolerance in the initial experiments was measured as the temperature at which half the test animals became inactive. Temperature was raised slowly, one degree every five minutes. Low temperature tolerance was measured similarly.

In subsequent assays, copepods were tested individually and their times and temperatures of collapse and their times of recovery at room temperature noted.

TABLE I

Times to succumb (TS) and times to recover (TR) of individual copepods subjected to sequential temperature changes and their correlations.

Traits	Obs.	Mean ± S.E. Minutes
1 = TS, slow increase 25–35° C	26	44.0 ± 0.8
2 = TR at 25° C, from 35° C	26	12.3 ± 1.3
3 = TS, slow decrease 25–3° C	10	36.1 ± 0.8
4 = TR at 25° C, from 3° C	10	3.5 ± 0.5
5a = TS, 34.5° C shock, no prior cold exposure	10	4.9 ± 0.7
5b = TS, 34.5° C shock, with prior cold exposure	10	6.1 ± 0.6
6a = TR at 34.5° C, no prior cold exposure	10	9.4 ± 2.4
6b = TR at 34.5° C, with prior cold exposure	10	13.8 ± 4.0

Correlations*	2	3	4	5a	5b	6a	6b
1	−.66	0.58	−.36	0.06	0.50	−.50	−.47
2		−.13	0.09	0.14	−.66	0.42	0.37
3			−.27		0.42		−.39
4					0.02		0.36
5a						−.65	
5b							−.39

* With 10 observations, correlations of 0.58 and higher are significant ($P < 0.05$).

In all the tests where temperature was raised gradually the upper limit of tolerance was around 35° C, regardless of test salinity and osmotic acclimation. Thus a more discriminating assay was needed.

Following the work of Battaglia (1967) on recovery from osmotic shock, the assay finally used was based on the time to succumb (TS) and time to recover (TR) from a temperature shock of 34.5° C (just below the critical temperature noted above). For this assay single individuals in 2 ml water in shell vials were placed in the aquarium kept at a constant 34.5° C, using the heating-stirring unit. The assay continued for 30 minutes. Those animals which had not recovered at the end of the assay were given a TR score of 30-TS minutes. The times to succumb and times to recover were combined in an index (30 + TS − TR) for ease of interpretation. This index and the alternatives to it are discussed further in the results section.

Salinity was measured using a refractometer, each measurement being checked by at least one other observer. Animals were acclimated to various salinities for 24 hours, except where noted.

RESULTS

Temperature tolerance measured by the succumb-recovery method

In the initial experiment, 26 animals were exposed to a gradually increasing temperature (to 35° C), half of those recovering (10) to gradually decreasing temperature (to 30° C) and all of them, subsequently, to a shock of 34.5° C. The times to succumb (TS) and recover (TR) were observed in all cases, recovery being at room temperature (25° C). The means and standard errors of the eight measurements and the correlations among them are shown in Table I.

TABLE II

Times to succumb (TS) and times to recover (TR) from temperature shock of two populations of E. affinis *acclimated for 24 hours at four salinities.*

Populations	Salinity ‰							
	3		9		12		15	
	Mar	Aug	Mar	Aug	Mar	Aug	Mar	Aug
TS minutes	3.1	4.2	3.5	4.3	2.8	6.0	5.3	13.3
TR minutes	18.9	13.2	8.3	5.9	12.6	4.7	3.1	1.6
Number of obs.	19	19	19	19	10	10	10	10

	F values and significances	
	TS	TR
Between populations	14.6**	11.1**
Between salinities	12.4**	20.6**
3 *vs.* 9, 12, 15‰	6.0*	52.3**
9 *vs.* 12, 15‰	13.0**	1.0†
12 *vs.* 15‰	18.3**	8.4**
Interaction	5.0**	1.1†

* $P < 0.05$
** $P < 0.01$
† Not significant
Standard deviations within subclass were 3.6 minutes (TS) and 6.9 minutes (TR)

As expected, animals resisting the shock for the longest period tended to recover earlier. The 12 correlations between the various TS and TR are negative, with two exceptions. The average correlation between TS and TR is −0.34; and between TS and the TR immediately following is −0.49. The 4 correlations between times to succumb are all positive, averaging 0.39, as are those between times to recover, averaging 0.31. Thus there appears to be consistency, not only between times to succumb and recover but also between high and low temperature tolerances. At least there does not seem to be a negative correlation between tolerance to high and to low temperatures, as might have been expected. And finally, cold shock did not significantly affect subsequent tolerance to high temperature shock.

Strain differences influenced by salinity with acclimation

Descendents of animals collected in March 1973 from the Patuxent River (Maryland) were compared with progeny of a sample collected further upstream in August 1973. Tests were done in the salinities at the collection sites (6‰ for March and <1‰ for August) and also after acclimation in the other salinity. The critical upper temperature, when raised gradually, was slightly higher for August 0–1‰ than for March 6‰ (35.7, 34.4° C), and for August in 6‰ than for March in 0–1‰ (35.5, 34.1° C). Average times of recovery (at room temperature) were 5, 6, 9, and 15 minutes, respectively. Thus the August population

TABLE III

Times to succumb (TS) and times to recover (TR) from temperature shock of two populations of E. affinis *at two salinities without acclimation.*

Population	Salinity ‰			
	0–1		12	
	Mar	Aug	Mar	Aug
TS min	2.9	3.1	3.4	5.2
TR min	20.3	13.4	12.2	9.5
Number of obs.	10	10	10	10

	F values and significances	
	TS	TR
Between populations	14.3**	2.4†
Between salinities	24.1**	3.8†
Interaction	9.1**	0.5†

** $P < 0.01$

† Not significant

Standard deviations within subclass were 0.8 minutes (TS) and 9.7 minutes (TR)

seems more tolerant to high temperature at both salinities. And tolerance seems greater in the salinities in which the samples were originally collected. While there is a suggestion of a strain difference in these results, they imply again that temperature of inactivation is not a useful measure and also that the tests should be done on individuals.

Individuals from the two populations acclimated to four salinities were tested using the shock-recovery assay. Days and salinities are partially confounded in the analysis, but at any salinity the results were repeatable between days. The results are shown in Table II. Clearly there were strain (population) differences and salinity differences in both time to succumb (TS) and to recover (TR); and salinity enhanced the strain difference, especially in TS. According to the means and the analyses of variance the critical change in salinity was between 9 and 15‰ for TS and between 3 and the higher salinities for TR. Whether measured by TS or TR, raising the salinity from 12 to 15‰ greatly increased the tolerance of both populations. Note that the effect of salinity was more systematic on the August population than on the March population. Tests were not done at salinities higher than 15‰ since the animals became almost inactive, although their behavior at 15‰ seemed quite normal.

Some of the animals tested at 15‰ did not succumb during the 30 minute assay period. These were given arbitrary scores of 29 minutes and 1 minute for TS and TR, respectively. The assay at 15‰ was repeated with the same animals at a higher temperature (36° C). The means for TS were 4.2 (March) and 5.0 minutes (August) and for TR were 14.3 (March) and 3.0 minutes (August). The difference between populations in TS was not significant ($0.05 < P < 0.10$). The difference in TR was significant ($P < 0.01$).

Table IV

Relationships between time to succumb and time to recover in two populations of E. affinis *with and without salinity acclimation.*

	d.f.	Sum of Squares (TS)	Sum of Products	Sum of Squares (TR)	r
With salinity acclimation (3, 9, 12, 15‰)					
Populations/salinity	4	389.5	− 250.3	677.9	−0.49
Salinities	3	488.8	− 853.1	2904.4	−0.72
Within	108	1411.6	− 146.5	5076.7	−0.05
Total	115	2289.9	−1230.9	8659.0	−0.27
Without salinity acclimation (0–1 and 12‰)					
Populations/salinity	2	16.4	− 31.1	274.5	−0.46
Salinities	1	16.9	− 78.1	360.0	*
Within	36	23.8	− 13.9	3416.6	−0.04
Total	39	57.1	− 123.1	4051.1	−0.26

* With one degree of freedom the correlation is trivially −1.

Strain differences influenced by salinity without acclimation

When animals were tested at salinities of 0–1‰ and 12‰, without acclimation, the effects of salinity and population on TR were not significant (Table III). Salinity and population still had a significant effect on TS. The differences between populations were in the same direction but smaller than in the tests after salinity acclimation. The effect of salinity acclimation was greater in the August population than in the March population.

The relationship between TS and TR

As was the case in an earlier experiment (Table I) the correlations between times to succumb and times to recover tended to be negative. We can now consider in more detail the relationships between TS and TR in the experiments reported above. The sums of squares for TS and TR, the cross-products and the associated correlation coefficients are shown in Table IV. All the correlation coefficients, whether between population means, salinity means or for individuals within classes, are negative. The correlations between population mean TS and TR are shown within salinity, since with only one degree of freedom between populations overall the correlation must be +1 or −1. There is remarkable similarity between the correlations in the experiments with and without salinity acclimation. The largest correlations are between the mean TS and TR of populations at each salinity (see Tables II and III for the means).

Indices of temperature tolerance

To express temperature tolerance as a single value, it seemed reasonable to combine TS and TR into an index. While TS and TR are related, as discussed

TABLE V

Indices of temperature tolerance of two populations of E. affinis
(Index = 30 + TS − TR).

	Salinity ‰ (with acclimation)			
	3	9	12	15
March population	14.2	25.2	20.2	32.2
August population	21.0	28.4	31.3	41.7
	Salinity ‰ (without acclimation)			
	0–1		12	
March population	12.6		21.2	
August population	19.7		25.7	
	F values and significances			
	With acclimation		Without acclimation	
Between populations	21.4**		3.5†	
Between salinities	26.9**		5.5*	
3 *vs.* 9, 12, 15‰	54.4**			
9 *vs.* 12, 15‰	6.4*			
12 *vs.* 15‰	20.0**			
Interaction	1.3†		0.2†	

* $P < 0.05$
** $P < .0.01$
† Not significant
Standard deviations within subclass were 7.9 minutes (with acclimation) and 9.8 minutes (without acclimation)

above, they are not so closely related that inclusion of both in an index is redundant. Four alternative possibilities are listed; I Rank on TS, then on TR; II Sum the rankings, downward by TS and upward by TR; III 30 + TS − TR, 30 added to keep index positive; IV $100 + (\overline{TR} \times TS) - (\overline{TS} \times TR)$, inversely weighting by means and adding 100 to keep index positive.

These indices can be compared on a limited scale using the data from the animals shocked twice in 15‰, as described earlier. An effective index of tolerance should result in somewhat similar rankings of the animals in both assays. Using each of the above indices in turn, the relative tolerances at each shock temperature are quite similar. Selecting the top 10 animals (of 20) within strain at each temperature shock we find that using index I or II, five animals are selected in both assays; and using III or IV, six are commonly selected. Thus there is no difference between the indices in this limited test of them. Index III seems preferable since it is a simple function of TS and TR. The variance of this index is also a simple function of the variances and the covariance between TS and TR. Since the covariance is subtracted and is negative, the index variance actually exceeds the sum of the variances of TS and TR. The more variable component contributes more to the index variance and so to the discrimination, which is appropriate if the variance reflects innate differences between animals. One could of course find an index to maximize strain differences over all the assays, but even then it might apply only to the current data.

In a later experiment 12 animals were tested and then re-tested after a period of about one hour at room temperature. The top five animals were the same in both assays and the correlation (repeatability) between the two index III values was 0.83. Consequently, one might infer that the index is measuring an inherent property of the animal quite accurately. The repeatabilities for times to succumb and to recover were 0.62 and 0.75, respectively, both below 0.83. One of the 12 animals did not succumb on either occasion. The average index was slightly higher in the second assay, so shocking the animals did not appear to decrease their tolerance.

Using index III (30 + TS − TR), the analyses shown in Tables II and III were repeated and the results are in Table V. Trends already noted are now clearer. There is a systematic increase with salinity in tolerance of the August population. The distinction between populations in 12‰ is greater following salinity acclimation. The advantage of the index over either TS or TR alone is suggested by the greater proportion of total variances attributable to population differences in the assays following salinity acclimation (compare F values in Tables II and V). In the assay without acclimation, the distinction using TS alone is greater (Tables III and V).

The results of re-analysis of the data from 36° shock at 15‰ (acclimated) are not shown in Table V. The index values were 19.9 (March) and 32.0 (August) and the difference is significant. As with the other assays on acclimated animals, the proportion of variance removed by populations is greater with the index than with TS or TR alone. Thus in general the index seems to enhance ascertainment of thermal tolerance, at least between the two populations used in this study.

Lower temperature tolerances

Low temperature tolerances of the March and August populations were investigated to a limited extent, using a temperature shock at 2.5°C. At 3‰ salinity (acclimated), means of TS were 3.4 (March) and 2.6 (August) and of TR were 6.7 (March) and 4.7 (August). At 9‰, four of the ten March animals did not succumb. The average TS and TR of the remainder were 6.1 and 5 minutes, respectively. None of the August animals succumbed at 9‰. Thus there appear to be strain differences at 9‰ for lower tolerance. The difference between populations tested in 3‰ were not significant. Increasing salinity seems to widen the range of tolerance as well as increase the upper limits of temperature tolerance.

DISCUSSION

The experiments described clearly show how salinity modified the effects of temperature. The results agree with other cases reviewed by Kinne (1964) where resistance to temperature extremes increased with salinity. The classic work on this subject is that of McLeese (1956) with the lobster *Homarus americanus*. He found systematic increases in upper lethal temperature with increase in salinity and also with increasing acclimation temperature and oxygen concentration. Other examples of the effects of salinity on temperature sensitivity are given by Schlieper in Remane and Schlieper (1971), including work by Ranade (1957) on the copepod *Tigriopus fulvus* showing a continuous rise in lethal temperatures as a function of salinity. Thus there seems to be quite general agreement on the enhancement of temperature tolerance by increasing salinity, at least in the range below stressful salinities.

The effect of salinity acclimation was more marked in the August population, resulting in a greater distinction between the populations following acclimation. Also there was a differential effect of increasing salinity resulting in increasing distinction between the populations at higher salinities. This suggests that since strain differences are enhanced, individual differences in tolerance may be enhanced at higher salinities. One way to determine this would be to select for tolerance at more than one salinity.

It should be emphasized that what has been shown by the data reported here is a repeatable, salinity-dependent difference in temperature tolerance between two populations of *Eurytemora,* which were collected originally in March and in August. I have not demonstrated that there is a difference in tolerance in winter and in summer. To establish such a difference the effects of other variables, including salinity, must first be removed. We have preliminary evidence that temperature acclimation has a significant influence on temperature tolerance.

The question of the nature of seasonal differences in temperature tolerance, if they exist, therefore remains open. The adaptive strategy of the species may well be a combination of genetic and physiological adjustment. I now discuss the curious discrepancy between our results on temperature tolerance and the distribution of *Eurytemora* in the wild.

As suggested by the title of the paper, the effect of salinity on temperature tolerance is not consistent with the observed distribution of *Eurytemora* in the Chesapeake Bay region. During the warm summer months it is confined to the upper fresher reaches of rivers around the bay. We collected samples at 28° C in nearly fresh water in August. This temperature is near the upper tolerance level, at least in laboratory culture—I could not culture *E. affinis* at 30° C, nor could Heinle (1969a). Yet we found thermal tolerance was greatest in 15‰, a salinity beyond the range in which the species is normally found at any season. So why should the general increase in salinity with rising average temperature result in a retreat of the species to areas of lower salinity? The inference is that there is no cause-effect relationship between salinity and the distribution of *E. affinis,* so other explanations must be invoked.

One factor contributing to the observed distribution might be increased predation in the summer and fall. This seems unlikely since Heinle (1970) found the density of *Eurytemora* was less affected by predation than that of *Acartia tonsa* (Dana). So unless there is selective predation, other species should be affected at least as much as *E. affinis.*

A more likely factor influencing the distribution of *Eurytemora* is competition, especially with the dominant summer species, *Acartia tonsa.* According to Heinle (1969a, Fig. 10) the growth rate and productivity of *E. affinis* is equal to that of *Acartia* at 12° C but only half as great at 25° C. Furthermore the density of *Acartia* may be closely related to phytoplankton production, whereas the density of *E. affinis* is not, resulting in additional competitive advantage to *Acartia* in the summer (Heinle, personal communication).

I wish to thank Joanne Janyska for collecting the data, Frank Hanson for critically reading the manuscript and Donald Heinle for advice on rearing the cultures. The work was supported by NSF Grant GA 33628.

SUMMARY

1. Thermal tolerances of populations of *Eurytemora affinis* were measured using two basic methods, at various salinities with and without acclimation. Little distinction in tolerances was made using temperature of inactivation. A more useful assay was temperature shocking at 34.5° C, observing time to succumb (TS) and time to recover (TR) over a 30 minute period.

2. Using the shock-recovery assay, there were repeatable and significant differences in tolerance between populations collected in March and August and also between salinities. Average tolerances and differences between populations generally increased with salinity.

3. The distinction between populations in thermal tolerance was greater when the animals were osmotically acclimated for 24 hours.

4. A simple index of tolerance combining TS and TR was suggested. In most cases the proportional variance between populations, using the index, was increased over TS and TR.

5. The seasonal distribution of *E. affinis* is contrary to that expected from the thermal tolerance-salinity relationship reported here. The explanation offered is that other species, for example *Acartia tonsa,* are at a competitive advantage during the summer and fall because of faster growth rate and greater dependence on phytoplankton.

LITERATURE CITED

BATTAGLIA, B., 1967. Genetic aspects of benthic ecology in brackish waters. Pages 574–577 in H. W. Lauff, Ed., *Estuaries.* American Association for the Advancement of Science Publication Number 83.

HEINLE, D. R., 1969a. Temperature and zooplankton. *Chesapeake Sci.,* **10**: 186–209.

HEINLE, D. R., 1969b. Culture of calanoid copepods in synthetic seawater. *J. Fish. Res. Board Can.,* **26**: 150–153.

HEINLE, D. R., 1970. Population dynamics of exploited cultures of calanoid copepods. *Helgolander Wiss. Meeresunters,* **20**: 360–372.

KINNE, O., 1963. The effects of temperature and salinity on marine and brackish water animals. I. Temperature. *Oceanogr. Mar. Biol. Ann. Rev.,* **1**: 301–340.

KINNE, O., 1964. The effects of temperature and salinity on marine and brackish water animals. II. Salinity and temperature salinity combinations. *Oceanogr. Mar. Biol. Ann. Rev.,* **2**: 281–339.

KINNE, O., 1967. Physiology of estuarine organisms with special reference to salinity and temperature, general aspects. Pages 525–530 in H. W. Lauff, Ed., *Estuaries.* American Association for the Advancement of Science Publication Number 83.

KINNE, O., 1970. *Marine Ecology,* Volume 1. Wiley-Interscience, 1774 pages.

MCLEESE, D. W., 1956. Effects of temperature, salinity and oxygen on the survival of the American lobster. *J. Fish. Res. Board Can.,* **13**: 247–272.

RANADE, M. R., 1957. Observations on the resistance of *Tigriopus fulvus* to changes in temperature and salinity. *J. Mar. Biol. Ass. U.K.,* **36**: 115–119.

REMANE, A., AND C. SCHLIEPER, 1971. *Biology of Brackish Water.* John Wiley & Sons, Inc., New York, 372 pages.

VERNBERG, W. B., AND F. J. VERNBERG, 1972. *Environmental Physiology of Marine Animals.* Springer-Verlag, New York, 346 pages.

Reference: *Biol. Biol.*, **148**: 35–48. (February, 1975)

BIOLOGY OF *NEOCHILDA FUSCA* N. GEN., N. SP. FROM THE NORTHEASTERN COAST OF THE UNITED STATES (PLATYHELMINTHES: TURBELLARIA)

LOUISE BUSH

Department of Zoology, Drew University, Madison, New Jersey 07940

A dark brown acoel has been collected for a number of years in bottom mud samples from Great Harbor and Buzzards Bay near Woods Hole, Massachusetts for use in class and experimental work. In the summer of 1965 I found a large population of the same species living intertidally in the salt marsh bordering Barnstable Harbor near West Dennis, Massachusetts. Since that time I have collected this species at a number of locations on the East Coast from Portsmouth, New Hampshire to Sandy Hook, New Jersey. Because of the presence of two distinct and conspicuous male reproductive organs at the posterior end of the animals it was natural for investigators to equate this species with *Childia spinosa* which was collected and described by Graff during his visit to Woods Hole in 1907 (Graff, 1911). Since *C. spinosa* has been shown to be the same species as *C. groenlandica* (Levinson, 1879) (see Hyman, 1959), several workers have recently published accounts using that designation (personal communication and Henley, 1968 and 1974; Costello, Henley and Ault, 1969; Boyer, 1971). However, a careful study of the anatomy of these brown acoels shows that they do not possess cuticular stylets as in *C. groenlandica* but, on the contrary, have eversible penes that preclude their being placed in the genus *Childia* at all. In fact it is necessary to set up a new genus and I am therefore describing them as *Neochildia fusca* n. gen., n. sp. and am presenting the data not previously published which I have obtained concerning their ecology and life history. It may also be noted here that, in spite of repeated efforts and extensive collecting, I have not been able to find specimens of *C. groenlandica* at the type locality (for *C. spinosa*) nor any place else along our coast, nor have I found anyone else who has seen it. It would be of great interest to obtain specimens that correspond to Graff's description for study.

MATERIALS AND METHODS

Materials for this study were secured in two ways: (1) by taking samples of substrate in sand or muddy sand areas along marsh streams; (2) by grab sampling of muddy bottoms in bay areas near marshes. Samples were brought in to the lab and allowed to stand for several hours or overnight, by which time the animals would have come up to lie on the surface and could be picked up with a medicine dropper and transferred to small syracuse dishes together with a little of the substrate and the associated organisms to serve as food. Many specimens kept in such dishes survived for weeks although egg laying usually ceased after a few days. Young hatched from eggs laid in such cultures were raised for up to two months until the cultures had to be abandoned.

The life history was studied by making regular monthly collections at the West Dennis, Massachusetts salt marsh which was relatively easy of access throughout the year. Half-pint plastic containers were filled with the upper 1–2 cm. of sand along the stream where the animals were known to occur. The specimens were counted and measurements of length were made as they moved along in a large drop of water on a slide. Slight flattening of the animal by a coverslip after measuring allowed a determination of sexual condition and general maturity to be made. Collections in the winter had to be made by taking trash along the deeper parts of the stream where the animals had migrated from the sand. Many grab samples taken at various times as well as the experience of the supply department at the Marine Biological Laboratory gave additional data for comparison with that obtained at the marsh.

Many specimens were studied alive with ordinary light microscopy and with phase contrast. Most whole mounts and serial sections were prepared by flooding with Bouin's fixative at room temperature although some other methods were also employed for comparison, such as fixation with glutaraldehyde, freezing with CO_2, *etc.* Whole mounts were made of both stained and unstained animals both with and without bleaching. The most satisfactory stain for whole mounts was found to be either Grenacher's borax carmine or Riser's stain (Jones, 1966, page 245 F39, page 269 F177). Sections were made in paraffin at 8 μ and stained with hematoxylin and eosin, Delafield's hematoxylin and eosin, Mallory's triple stain or special stains for particular facets of the histology.

Observations, Results and Discussion

Systematic section

Neochildia n. gen.

The structure of the penes in *N. fusca* clearly distinguishes this species and makes it necessary to set up a new genus. The fact that the penes are inverted into the seminal vesicles as short straight tubes with some granular material at the proximal end is somewhat similar to the condition in several genera of the family *Convolutidae* as defined by Dörjes (1968), *e.g.*, *Pracaphanostoma*, *Pseudaphanostoma*, *Faerlea*. However, in the description of none of these do I find any mention of the crown of muscle fibers half way along the penes or of any similar mechanism by which the lower end of the penis is spread open to allow of the eversion of only the proximal half. The plesiomorphy here seems to me to be the eversibility of the penes which is characteristic of the Family *Convolutidae*: in the crown of muscles and type of eversion the genus *Neochildia* has evolved an apomorphic character distinctive for this genus. The two complete sets of copulatory organs constitute a good generic character, but the doubling of organs is not unusual in Turbellaria and does not have phylogenetic significance. The presence of unusually dark pigment may or may not be a genetic character. The definition of the new species must serve also as the definition of the genus until more species are discovered which belong here.

Neochildia fusca n. sp.

TYPES. Holotype, a longitudinal serial section from Buzzards Bay Massachusetts, United States National Museum (USNM) Cat. No. 52011; paratypes, whole mounts and serial sections from several localities, USNM Cat. Nos. 52012–52035 and in the author's collection.

DEFINITION. Shape elongated oval. Color dark brown with orange rhabdite packets. Length 4–6 mm. Brain internal with typical statocyst. Frontal organ not well developed. Mouth ventral without pharynx. Well developed body wall musculature with outer circular, diagonal, longitudinal and inner circular layers. Outer parenchyma dense and not vacuolated. Ovaries and testes paired, testes dorso-lateral to the ovaries and sometimes meeting in center behind the brain. Mature dark brown eggs lie dorsal and immediately posterior to the mouth. No female accessory organs. Two male copulatory organs opening separately into a shallow, ciliated atrium with genital pore slightly dorsal on the posterior end. Walls of copulatory organs thick, fibrous, with 10–12 longitudinal muscles spaced within the circular fibers. Penes eversible, with crown of small muscles attached half way along and acting, together with associated muscles in the parenchyma, to open the distal end of the penes when the proximal end is everted.

HABITAT. Mud or sandy mud at depths to 10 m all year; in summer it appears intertidally in brown, orange, or purple surface build-up on sand or sandy mud along salt marsh streams; in salinities from 2.2–35‰.

COLLECTIONS. Portsmouth, New Hampshire, intertidal sandy mud behind Wentworth Hotel each year in June 1968–72 and in August 1973; West Dennis, Massachusetts, in marsh stream north of Aquaculture Corporation Laboratory throughout the years 1965–74; also in Massachusetts in bottom mud of Buzzards Bay, in the Pocasset River, in marsh in Menemsha Harbor and in Provincetown Harbor, various times; New Jersey, Sandy Hook State Park Wildlife Sanctuary, Septembers 1966–70.

Description

COLOR. Medium orange brown in immature individuals to dark chocolate brown in adults.

SIZE. Newly hatched 0.2–0.35 mm; at first evidence of reproductive organs 1.2–1.3 mm; older animals to about 4.5 but a few to 6 mm.

SHAPE. Oval with broadly rounded anterior and slightly narrower rounded posterior; body thick to ∽0.5 mm, with convex dorsal and slightly concave ventral sides; lateral edges at times somewhat enrolled. Often during spawning season the posterior 1/6 of the body may be suddenly narrowed, extended, and uptilted and then as briefly withdrawn. In this part the sperm filled seminal vesicles can be seen as two light colored ovals. For the significance of this see the discussion of reproduction and spawning.

STRUCTURES. Due to the very dark brown color many details cannot be distinguished in the living animals. In flattened animals with phase or light microscope the following can be made out (Fig. 1).

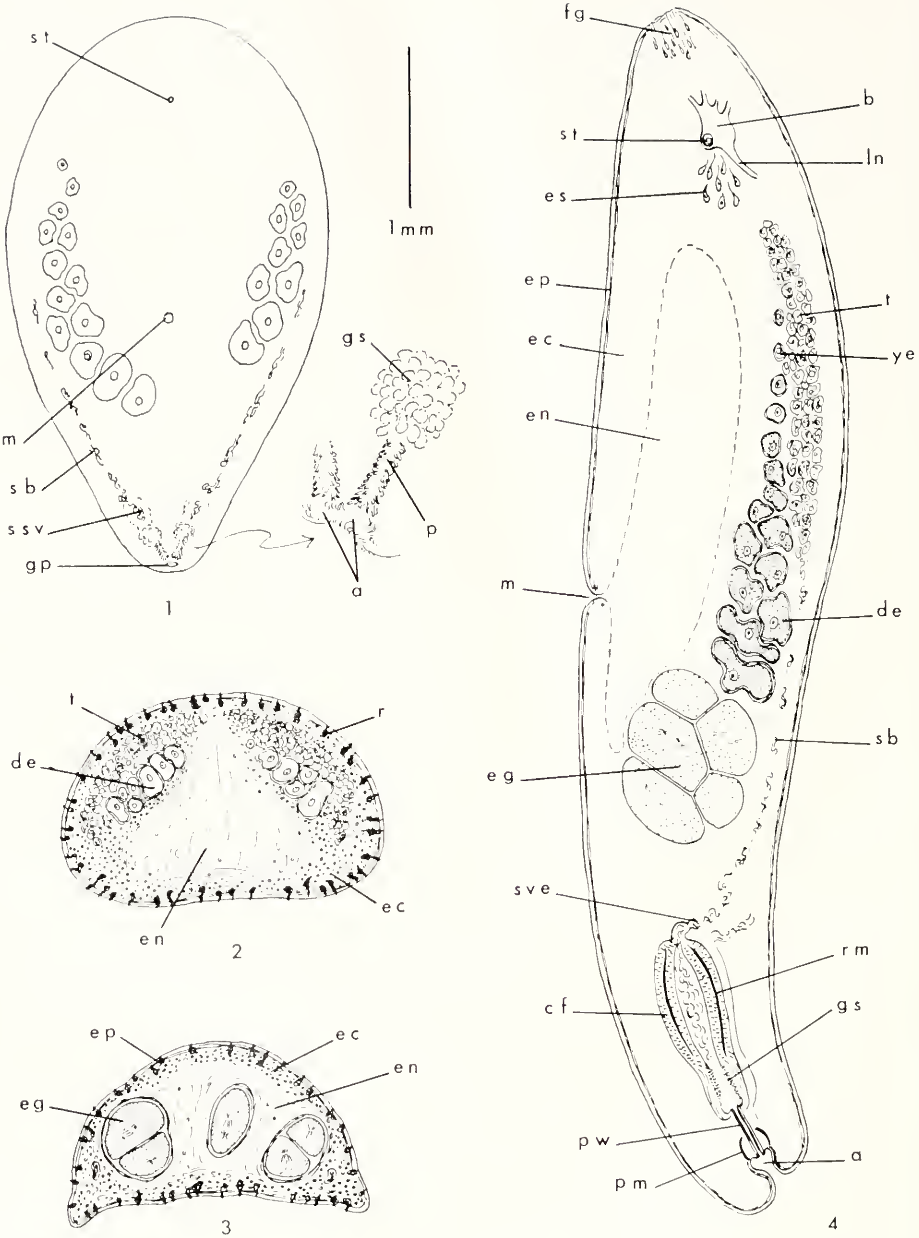

st
1 mm
m
sb
ssv
gp
gs
p
a
1
t
r
de
ec
en
2
ep
ec
en
eg
3
fg
b
st
ln
es
ep
ec
en
t
ye
m
de
sb
eg
sve
rm
cf
gs
pw
a
pm
4

FIGURE 1. Living *Neochildia fusca*, slightly flattened. Abbreviations used are: a, atrium; b, brain; cf, circular fibers; de, developing eggs; e, epithelium, es, eosinophilic glands; ec, ectocytium; en, endocytium; ep, outer layers of body wall; fg, frontal gland; gs, granular secretion; gp, genital pore; p, penis; pm, protractor muscle; pw, penis wall; r, rhabdite gland; rm, retractor muscle; sb, sperm in bundles; ssv, sperm in seminal vesicle; st, statocyst; sve, seminal vesicle entrance; t, testis; ye, young eggs.

Cilia over the entire body; 5 μ long on dorsal side, 7–8 μ on lateral and ventral sides; isolated longer cilia scattered over body to 12 μ.

Color present both as dark brown granules in the outer epithelium and as orange color in solution and as orange rhabdite packets. Each rhabdite packet is made up of rice-grain-shaped 2.5 μ long bodies which may protrude on the surface as orange papules. Each packet is the product of a large gland cell the main part of which lies inside the epithelium. The glands and thus the associated papules are scattered at fairly regular intervals but are not arranged in regular rows. They are most numerous on the posterior third of the body on both dorsal and ventral sides. Over the rest of the body there are more on the dorsal than on the ventral side and at the very anterior end none occur in a small area where sections indicate there may be openings from a kind of frontal gland.

Mouth without a pharynx, lying about 3/5 of the way back from the anterior end.

Statocyst in the midline about 1/6 of the way from anterior end. Diameter in fixed mature specimens 25–30 μ. One statolith, a slightly flattened sphere.

Female reproductive system without accessory ducts or bursa. Developing eggs can be seen arranged in irregular rows down each side of the body with the largest just posterior to the level of the mouth. Eggs are dark brown and entolecithal.

Male reproductive system: in flattened animals during the spawning season mature sperm can be seen in light colored, coiled, irregular clumps extending from about the level of the largest eggs to the two male reproductive organs occupying the posterior 1/6 of the body. Seminal vesicles appear as light oval areas on each side with clusters of refractile granules and penes extending almost to the genital pore which is located slightly dorsal to the tip of the posterior end. Just inside the pore there is a shallow atrium into which the penes open separately. Testes are not to be distinguished clearly except in sections.

Findings from sections

BODY LAYERS. The epithelium consists of cells whose outer parts form a homogenous appearing surface layer and whose inner ends with the nuclei are insunk below and between the underlying muscle fibers. The homogenous layer (epicytium of some authors) (Hyman, 1959) contains brown pigment granules in a distinct outer band as well as scattered throughout the cell bodies. In some sections the band appears double with one line of granules near the surface at the base of the cilia and another deeper and thicker layer inside this. The epicytium is penetrated by the necks of the rhabdite gland cells, the bodies of which lie at various depths in the underlying parenchyma. In sections prepared with Bouin's fixative and hematoxylin stains the glands appear as empty spaces 10–15 μ across and 25–30 μ long. With glutaraldehyde fixation and Mallory's triple stain the contents appear as a bubbly irregular bright blue mass. In these sections

---

FIGURE 2. Cross section through region of developing eggs and testes. Abbreviations as in Figure 1.

FIGURE 3. Cross section through region of mature eggs. Abbreviations as in Figure 1.

FIGURE 4. Diagram of a sagittal section of entire animal. Abbrevations as in Figure 1.

the secretions can be seen to protrude at the surface to form the papules mentioned above. In sections cut horizontally near the surface of the body the necks of the glands appear as irregular circular spaces (3–5 in diameter) in the epicytium. In addition to these, minute "pin holes" (0.5 across) are arranged in irregular lines and must mark the outlines of the epithelial cells. Their appearance under high magnification suggests that they represent the part of the cell walls not firmly attached to each other.

Muscle fibers are well developed and occur both in the outer "body wall" and as large dorso-ventral strands running across the body. The body wall muscle occurs in four layers: (1) a sparse outer layer of circular fibers running between the inner ends of the epithelial cells; (2) a layer of two sets of diagonal fibers which cross each other at right angles and which are either interwoven or very closely associated; (3) the thickest and most conspicuous layer, relatively heavy longitudinal fibers; (4) inner circular fibers which are very sparse over most of the body but increase greatly in number in the posterior end of the body. Nuclei and epithelial cell bodies as well as the rhabdite glands lie within the muscle layers and, toward the inside, parenchyma cells also occur, making this entire outer wall a firm unit. Additional fine muscle fibers are arranged in concentric circles around the mouth so as to form an oral sphincter.

At the posterior end of the body the longitudinal fibers converge toward the genital pore. In this area also both the longitudinal and circular layers are increased in number so that there is a meshwork of fibers running perpendicular to each other and spaced at intervals throughout the parenchyma around the copulatory organs.

The ectocytium (peripheral mesenchyme of Hyman 1959) and the endocytium (central mesenchyme of Hyman 1959) are clearly distinguishable (Figs. 2, 3). The ectocytium is a parenchyma with rather closely spaced nuclei, forming a firm packing around the inner layer of the body wall musculature, various gland cells and the parts of the nervous and reproductive systems. The endocytium, as in most acoels, contains scattered nuclei, lightly staining irregular cytoplasm and vacuoles with partially digested food and storage granules.

NERVOUS SYSTEM. The brain (Fig. 4) with the statocyst on its ventral side is located in the center of a cross section of the anterior end. Some eosinophilic gland cells occur in groups just posterior to the brain but their ducts, if any, could not be made out and the nerves leading from the brain are also difficult to distinguish in my sections. A large longitudinal posterior nerve proceeding dorsally on each side and at least two anterior nerves of good size are present.

At the anterior tip of the body certain cells in the parenchyma appear to have processes which run forward to penetrate the epithelium. The epithelium at this point is somewhat thinner, with shorter cilia and, as already noted, without rhabdite glands. These cells have nuclei similar to parenchyma cells, relatively small amounts of cytoplasm and only in certain sections do their processes (or ducts?) take any stain. Their exact nature has not been determined, but together they may be considered to form a sort of primitive frontal gland.

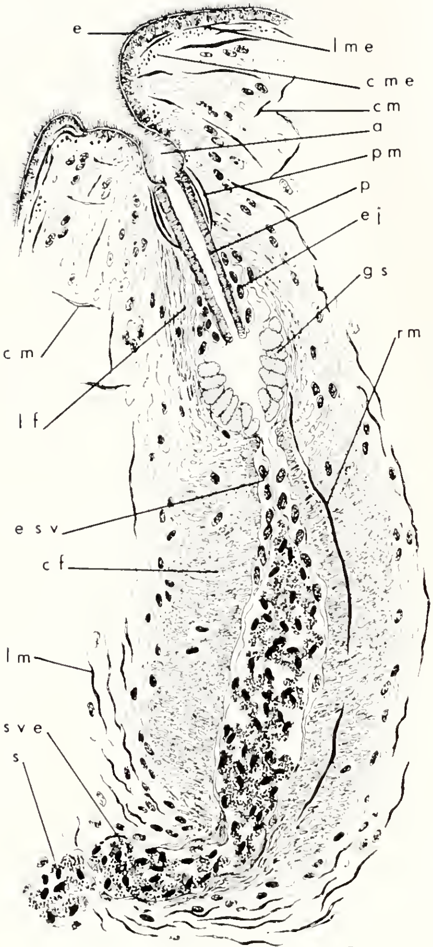
FEMALE REPRODUCTIVE SYSTEM. The first stages of egg development are found in the epicytium either a short distance behind the brain near the center or a little to each side at this level. Cells which can be distinguished as eggs by their enlarged nuclei and conspicuous nucleoli occur singly. Progressively later stages extend posteriorly in a line along the body just medial to the line of developing sperm. The cytoplasm in the young eggs contains some brown pigment and as the cytoplasm increases in amount the pigment also increases until the newly laid eggs appear the same dark color as the adult animals. Developing eggs are surrounded by a layer of nurse cells which disappear by the time the eggs are ready to be laid. Eggs ready to be laid lie in a closely packed group pushed into the endocytium just behind the mouth (Fig. 3). Such eggs usually show mitotic figures, but the complete cytology of their development has not been worked out.

Male reproductive system. Developing sperm occur in follicles which are packed together in compact testes lying dorso-lateral to the eggs (Fig. 2). In actively reproducing animals the testes extend from a point where they meet in the middle of the body just behind the brain to a point just anterior to the level of the mouth on each side. At other times and in younger animals they begin further back on each side of the body. Large generative cells are found scattered throughout. The sperm in each follicle are surrounded by a thin membrane and remain together as they leave the testes and move down the side of the animal to enter the seminal vesicles.

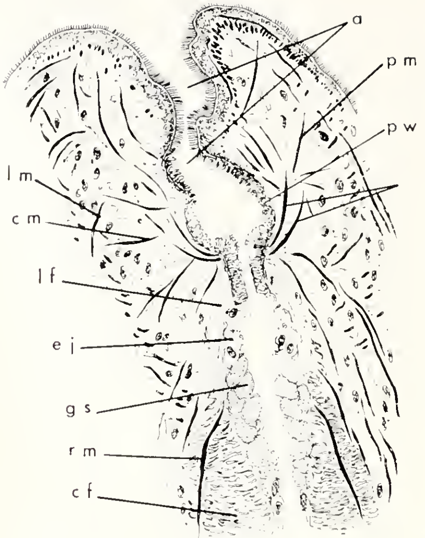
My observations of living sperm agree with the descriptions presented by Henley (1968 and 1974). In sections the follicles with sperm are conspicuous not only because of the surrounding membrane and the uniform condition of the sperm in each one but also because of the refractile bodies present in the later stages. The refractile bodies also make conspicuous the presence of individual sperm scattered throughout the tissues of many animals during the spawning season. In some specimens also large masses of individual sperm are found, often located at the anterior end of the animal and lying either just inside of or partly on the surface of the epithelium. Such a mass shows no signs of membranes around groups of sperm and is larger than any single follicle. Often individual sperm appear to be moving out of the mass into the tissues. Clearly these masses as well as the individual sperm in the tissues are foreign sperm deposited on the surface of the animal by a partner.

The two copulatory organs lying side by side in the posterior end of the body are each a separate entity opening separately into the atrium (Fig. 5). The atrium itself is lined with a pigmented epithelium like that of the exterior except that the cilia are somewhat longer here. Since the two copulatory organs open into it from the anterior the atrium is broad anteriorly and it is short, narrowing down rapidly to the genital pore, so that it is actually, when at rest, rather like a fat heart with the point directed to the outside. When the penes are protruded the genital pore opens and turns outward so that the atrium is partly obliterated.

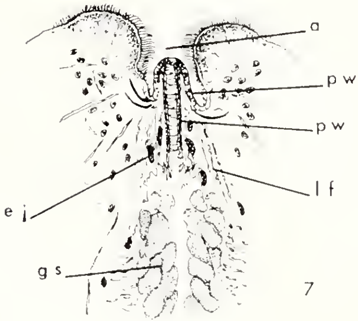
Each copulatory organ is pear-shaped with a short, curved, tube-like entrance extension at the anterio-lateral corner. The anterior and widest part of the organ proper comprises the seminal vesicle; the narrower posterior portion contains granular material and an eversible penis. The outer wall of the organ is composed of an epithelial lining and a thick (15–20 μ) layer of fibrous (muscular?) tissue, which is thickest around the seminal vesicle, and thinner around the penis


e
lme
cme
cm
a
pm
p
ej
gs
rm
cm
lf
esv
cf
lm
sve
s

5

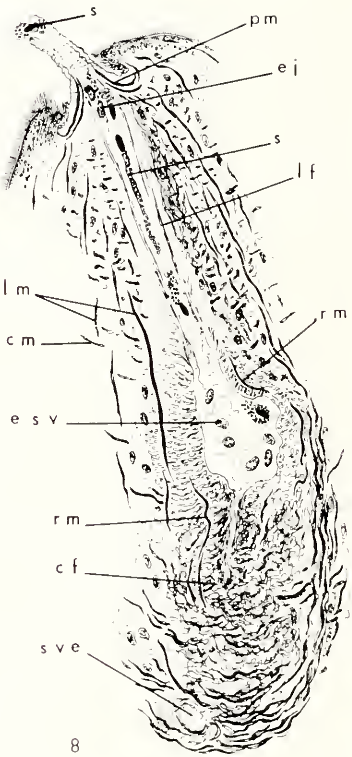

a
pm
pw
lm
cm
lf
ej
gs
rm
cf

6


a
pw
pw
lf
ej
gs

7

.1 mm


s
pm
ej
s
lf
lm
cm
rm
esv
rm
cf
sve

8

and at the anterior end. Around the seminal vesicle the fibers run circularly but toward the distal end around the penis the direction changes so that the fibers run longitudinally. Here they are also finer. Around the entrance tube the circular fibers gradually decrease in number until they disappear and there is no distinct end to the tube clearly marking it off from spaces in the surrounding parenchyma. The fibers are distinctly different in staining reactions from muscles in the surrounding body and from the few muscles that form part of the wall. These latter are a series of widely spaced thick muscle fibers, ten to twelve in number, which run longitudinally through the circular fibers of the wall of the seminal vesicle. The question of the true nature of the fibers needs to be studied by histochemistry. There are only a few scattered nuclei present in the wall among the fibers.

The epithelial lining of the seminal vesicle is very thin with scattered nuclei, except for the area at the distal end where it is thicker with a number of large nuclei. Just beyond the nucleated area toward the penis a large amount of granular material is found, the granules of which are clumped in a cell-like arrangement although no nuclei could be found in the clumps. In cross section the appearance of this area suggests a thick cellular epithelium lining the organ distally as far as the penis. Since no granular gland cells can be found elsewhere it is suggested that the large epithelial cells at the end of the seminal vesicle act as holocrine glands which break down to form more granular material after ejaculation has used up that already present. Around the penis itself a few more nuclei indicate the presence of more epithelial cells, some of which can be found in the lining of the penis when it is everted. In the living animal the granular material is evident as a mass of glistening clumps at the proximal end of the penis.

The penis itself is composed of very thin fibers, a thicker inner circular layer and a very thin outer longitudinal layer, which arrangement is reversed, of course, when the penis is everted. The entire penis is straight, about 80–100 μ long; its most conspicuous feature is the presence of a crown of short muscle fibers (Figs. 5, 6, 7, 8, and see also Henley, 1974 Fig. 6B) which are attached about half way along the inverted penis and which run outward from there to a point at the proximal end of the male atrium. At this point a number of fine muscles of the ectocytium also attach and radiate outward toward the edges of the body. Sections showing penes in various stages of eversion indicate that this complex of muscles acts as protractor for the penis. The crown of muscles attached to the penis, on contracting, draws the penis outward at the same time as the radiating muscles in the ectocytium pull the lower end of the penis tube open so that the proximal half can be everted and pushed out through the genital pore by the forceful contraction of the circular muscles of the posterior end of the body.

Figure 5. Penis and seminal vesicle in the resting state. Abbreviations used are: a, atrium; cf, circular fibers; cm, circular muscle in parenchyma; cme, circular muscle in body wall; ej, epithelium of ejaculatory duct; esv, epithelium of seminal vesicle; gs, granular secretion; lf, longitudinal fibers; lm, longitudinal muscle in parenchyma; p, penis; pm, protractor muscle; pw, penis wall; rm, retractor muscle; s, sperm; sed, sperm in ejaculatory duct; svc, seminal vesicle entrance.

Figures 6 and 7. Stages in eversion of penis. Abbreviations as in Figure 5.

Figure 8. Completely everted penis with empty seminal vesicle. Abbreviations as in Figure 5.

This squeezing action would also collapse the seminal vesicle and force the sperm out of the body. The longitudinal muscles in the seminal vesicle wall plus the effect of relaxation of circular and contraction of longitudinal muscles in the posterior end of the body would act as retractors. The sudden narrowing and extension of the posterior end of the body during spawning corresponds with this sort of muscle activity.

EGGS AND YOUNG. Egg masses measure about 1 mm in diameter and each contains from 7 to 40 eggs, each about 150 μ in diameter. Each egg is surrounded by a thin, tough, clear layer of jelly and the entire mass is further enclosed in a layer of sticky jelly, somewhat irregular on the outside and adhering tightly to the substrate though there is no formed stalk or attachment area. In the laboratory at 20° C development to hatching requires about 7 days. Newly hatched animals are oval in shape and have a statocyst and an outer layer of ciliated epithelium, but the parenchyma is not yet differentiated clearly into ectocytium and endocytium and there are no traces of reproductive organs.

Life history

A population of *N. fusca* in the marsh at West Dennis, Massachusetts was studied extensively because the area was easy of access and, being intertidal, allowed observations of the exact conditions under which the animals were living. Regular monthly collections were made in 1965–1966 and at less regular intervals for the next 8 years. Observations of the life cycles of other populations, particularly the collections made by grab sampler in Buzzards Bay correlate with the observations made at Dennis although there may be some minor variations in the time of events. The population in Buzzards Bay appears to be in all respects like that in the Dennis marsh.

The marsh at Dennis is built up on a sandy base between rows of sand dunes and the small stream which drains the marsh at low tide is completely under water at high tide. There is no fresh water runoff except immediately after a rain and the water in the stream measures at other times about 35‰. The upper part of the stream is formed by several meandering water courses and these, as well as the greater part of the main stream, are bordered by 1′–2′ high banks held in place by the roots of *Spartina* sp. which form a tangled mass collecting debris and fine silt in the water along the edges. The bottom of the stream is sand covered by a layer of fine brown sediment. Along the lower part of the stream it is wider with sloping banks that are uncovered but never completely dry at low tide. There is often a brown or purplish bacterial scum on the surface and in this and in the upper 2–3 cm of the sand bacteria, algae and a host of microscopic species including *N. fusca* are found in abundance. Similar locations in New Jersey, Martha's Vineyard, and New Hampshire have also yielded collections of this species.

In winter this exposed sandy area becomes completely barren of life. Several feet of ice may form on the vegetation away from the stream but my experience was that the stream itself remained open with only a thin film of ice on the surface. And in among the roots and trash in the water, although they were seldom found there in the summer, *N. fusca* was collected from December to April and early

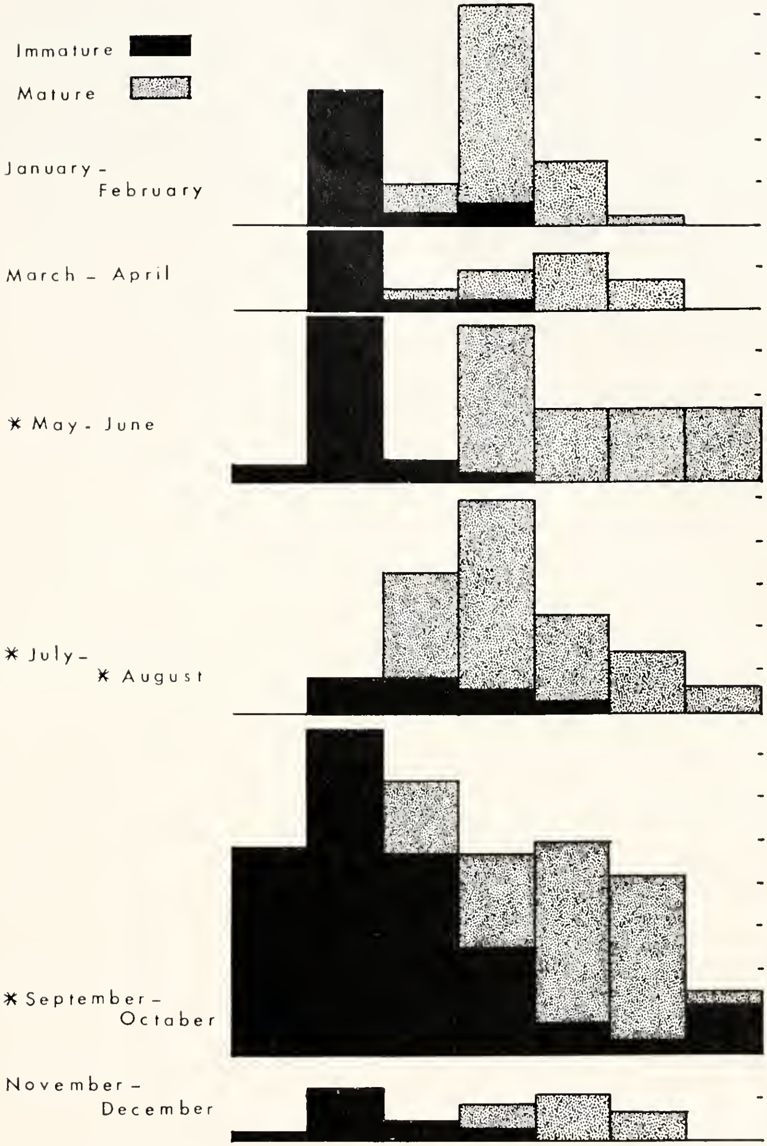


FIGURE 9. Annual life cycle of *Neochildia fusca*. The columns from left to right represent size groups: newly hatched –0.5 mm, 0.5–1.0 mm, 1.0–1.5 mm, 1.5–2.0 mm, 2.0–2.5 mm, 2.5 and above. Asterisk indicates spawning.

May. By the middle of May, when the summer populations were beginning to build up on the sand flats, *N. fusca* moved out from the stream and was to be collected in the sand. And in the middle of May the first animals were collected which laid eggs about 48 hours after being brought into the laboratory.

Figure 9 summarizes the annual cycle of the population at Dennis. Animals collected in pint samples of the top 2–3 cm of sand were measured and examined to determine their reproductive condition, and counted as belonging to the size groups in the graph. The population during the winter months, November to May, is made up of two groups: one, animals which go into the winter as adults and may have spawned already (the presence in the fall of sperm in the seminal vesicles and of young eggs suggests this); two, young animals which have hatched from the previous summer's spawning and are not yet mature. In May the members of the mature group laid eggs in the laboratory as noted and in the field newly hatched young began to appear in the collections in early June, which indicates that these older overwintering adults spawn at about the time when they move out of the stream and up into the sand flat. Shortly after this they evidently die off since I found that in early June spawning adults were very few in number, only a few, mostly spent, larger individuals being present. In the meantime the younger overwintering animals are maturing, so that by July and August they begin to spawn and many reproducing individuals are then present as well as newly hatched and young animals. Spawning continues into September, but with the onset of lower temperatures and shorter days further growth stops and the population, now made up of mature animals from the first spawning in May plus some from later spawnings who have matured and the young of all ages, move up into the stream to spend the winter in the debris at the edge of the pools there. Thus we have an annual life cycle with some animals probably spawning both in their first summer and then again the next spring before dying.

Behavior

Behavior of the animals when kept in small syracuse dishes in the laboratory was studied for up to two months after hatching as well as in individuals brought into the laboratory from time to time. Food was supplied by adding organisms from the collections in the field. Although activities observed in this way are not necessarily the same as they would have been in the field, they nevertheless indicate some things of interest.

N. fusca, although not a typically elongate interstitial animal, is nevertheless able to move through the substrate without difficulty as is attested by the fact that they come up to the surface of a bucket of mud or a dish of sand brought into the laboratory within a few hours. Their thick but flexible body with well developed body wall muscles and relatively solid parenchyma makes them seem much less fragile than many acoels. They move rapidly but smoothly and, on occasion as in feeding, can react very rapidly.

Feeding behavior is characteristic. Often in sand samples allowed to sit in the laboratory they were observed with the posterior end down between the sand grains and the anterior end raised slightly above the surroundings with the sides of the body slightly enrolled. Such a position makes them a perfect trap for passing animals. In a culture dish with less sand they lie quietly on the bottom, but in either case when potential prey appears a startling, quick, raising and then clamping down of the anterior end serves to pin down and hold the victim fast as it is pushed and sucked up through the mouth. Small crustacea, mostly copepods, as well as other turbellaria, nematodes and small annelids have all been found in the endocytium of mature animals. Animals up to about 1 mm long usually contain only diatoms, perhaps because of size, but also it may be because of inability on the part of the young animals to capture active prey.

Cannibalism may occur in crowded cultures or in ones without sufficient food, but an avoidance reaction on the part of the animals probably prevents this under normal conditions. Whenever one *N. fusca* runs head on into another *N. fusca* there is an immediate and sharp withdrawal followed by a change of direction and moving away. Sensory structures at the anterior end undoubtedly are effective here although, as pointed out before, a distinct frontal organ is not evident in this area. This reaction has obvious advantages in preventing cannibalism and its suppression during mating is striking.

Besides the suppression of the avoidance reaction the characteristic quick outward thrust of the posterior end also marks mating behavior. Two animals will be seen to meet and instead of jerking away from each other, to spend some time moving about over each other in an exploratory fashion. On one occasion I was able to observe this for some time and I noted that finally one of the pair of animals turned slightly away from the other, shot out the posterior end so as to touch the partner quickly and lightly and then moved away. The two animals were immediately fixed and sectioned and one animal of the pair shows in the sections both penes protruded from the genital pore and the seminal vesicles emptied of sperm. Similar reactions were also observed on other occasions. This behavior together with the patches of sperm on or in the epithelium of many animals, plus the fact that the penis is a thin walled soft-appearing organ leads me to the conclusion that impregnation is by means of a deposition of sperm on the surface. The glandular material associated with the penis and extruded with the sperm may well have an enzyme which aids the sperm in penetrating the epithelium.

Egg laying was not observed satisfactorily. The presence of light appears to inhibit this and some workers have found that a sure way of obtaining eggs is to place the animals for several hours in a dark place. My attempts to observe the process in the cultures only resulted in the finding that the egg masses were usually deposited very early in the morning. Early one morning, however, when the lights were turned on to start observations I observed one animal to be moving around and around in a tight circle in one spot in an unusual way. This kept up for about 10 minutes and when the animal moved away it left behind a small egg mass on that spot. The position of the mature eggs in the endocytium just dorsal to the mouth make it seem logical that they would exit through that orifice, although it is not impossible that they would be extruded elsewhere. The fact that eggs are sometimes extruded through the mouth when animals contract on being fixed, the lack of any signs of torn epithelium in the animal observed above and the behavior of that animal leads me to expect the mouth to be the point of exit.

This work was supported by the Systematics-Ecology Program (SEP) of the Woods Hole Marine Laboratory and by Drew University. I thank particularly Dr. Melbourne R. Carriker and the staff of SEP for their help. I am also grateful to Dr. Donald P. Costello and Dr. Catherine Henley for their encouragement and interesting discussion of many points. Also I thank Dr. E. Kirsteuer and the American Natural History Museum for the opportunity to study Dr. Hyman's slides of *Childia groenlandica*.

SUMMARY

1. *Neochildia* new genus is established on the basis of the presence of two separate and complete copulatory apparatuses, each with an eversible penis which has a crown of muscle fibers attached about half way along it. The type species, *Neochildia fusca*, although it has been used in a number of studies at the Marine Biological Laboratory and has previously been identified as *Childia groenlandica*, cannot be included in the genus *Childia* which is characterized by two copulatory organs but which is without any signs of eversible penes and has instead a bundle of cuticular stylets.

2. The anatomy and histology of *N. fusca* is described in detail. The most conspicuous field characteristic is the dark brown color which is unusual among acoels, and, indeed, among smaller Turbellaria in general.

3. The habitat of the new species is sand or sandy mud along marsh streams or in the bottom of associated shallow bays. The known distribution is from New Jersey to Maine along the East Coast of the U. S.

4. The life history involves the following annual cycle: an overwintering of mature or nearly mature animals subtidally or in the deeper waters of the marsh streams, an early spawning in May or June followed by a die-off of the overwintering animals, the maturing and spawning of the next generation from July to September and the overwintering of animals derived apparently from both the summer generations.

5. The food of young animals consists almost entirely of diatoms, but after the first few weeks they become highly carnivorous and are found to contain mostly copepods and other crustacea as well as worms and other Turbellaria.

6. Mating behavior is described and the conclusion is reached that sperm are deposited as a packet on the surface of the partner, not hypodermically injected. The very short contact between animals, the soft nature of the penes and the presence of masses of sperm entering through the epidermis in sections support this conclusion. Some evidence is presented that suggests that eggs are laid through the mouth.

LITERATURE CITED

Boyer, B. C., 1971. Regulative development in a spiralian embryo as shown by cell deletion experiments on the acoel, *Childia*. *J. Exp. Zool.*, **176**: 97–106.

Costello, D. P., C. Henley, and C. R. Ault, 1969. Microtubules in spermatozoa of *Childia* (Turbellaria, Acoela) revealed by negative staining. *Science*, **163**: 678–679.

Dörjes, J., 1968. Die Acoela (Turbellaria) der Deutschen Nordseeküste and ein neues System der Ordnung. *Z. Zool. Syst. Evolutionsforsch.*, **6**: 56–452.

Graff, L. V., 1911. Acoela, Rhabdocoela und Alloeocoela des Ostens der vereinigten Staaten von Amerika. *Z. Wiss. Zool.*, **99**: 321–428.

Henley, C., 1968. Refractile bodies in the developing and mature spermatozoa of *Childia groenlandica* (Turbellaria: Acoela) and their possible significance. *Biol. Bull.*, **134**: 382–397.

Henley, C., 1974. Platyhelminthes (Turbellaria). Chapter 5, pages 267–343 in A. C. Giese and John S. Pearse, Eds., *Reproduction of Marine Invertebrates I.* Academic Press, New York and London.

Hyman, L. H., 1959. Some Turbellaria from the coast of California. *Amer. Mus. Novitates*, **1959**: no. 1493.

Jones, R. M., 1966. *Basic Microscopic Techniques.* University of Chicago Press, Chicago and London.

Reference: *Biol. Biol.*, **148**: 49–59. (February, 1975)

ON THE DEVELOPMENT OF THE SEA-STAR, *ASTROPECTEN LATESPINOSUS* MEISSNER

MIÉKO KOMATSU

Department of Biology, Toyama University, Toyama 930, Japan

There have been several reports on the development of sea-stars belonging to the genus *Astropecten*. Newth (1925) reported on the early development in *Astropecten irregularis*, and Hörstadius (1939) gave a detailed description of the entire process of development in *Astropecten aranciacus*. Further, Mortensen (1921, 1937) outlined the form of bipinnariae of *Astropecten scoparius, Astropecten polyacanthus* and *Astropecten velitaris*. It has been known that all of these sea-stars form typical bipinnariae. The majority of the species of *Astropecten* undergoes metamorphosis without passing through the brachiolaria stage.

The present study was initiated to investigate the development of *Astropecten latespinosus*, which is one of the most common sea-stars in Japan. It becomes evident in the present study that the development of this species is somewhat different from that of the other species of *Astropecten*, in having a larva of barrel form and in undergoing metamorphosis very early.

In the present paper, the entire process of development in *Astropecten latespinosus* is described, especially the external morphology and the skeletal system. Developmental processes in the internal structure will be reported later.

MATERIALS AND METHODS

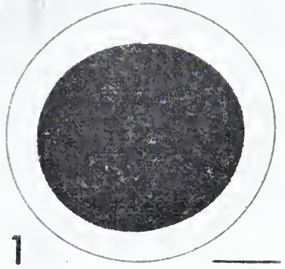
Adults of the sea-star, *Astropecten latespinosus* Meissner were collected on the coast of Toyama Bay, Sea of Japan, during the presumed breeding season, from the end of June through July in 1972 and 1973.

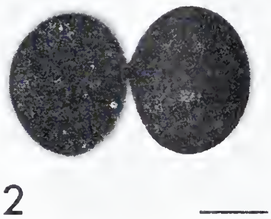
By treatment with 1-methyladenine of the ovaries which were removed from the females according to Kanatani (1969), fertilizable ova were obtained. Dilute sperm suspension was prepared from small pieces of mature testes and added to the petri-dish containing the ova. Embryos and juveniles were reared in glass vats at laboratory temperature of 25 ± 2° C.

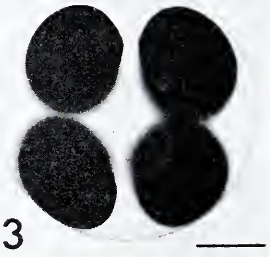
Observations were made with a dissecting microscope. Measurement of living embryos was performed with an ocular micrometer. Specimens were fixed at appropriate intervals with Bouin's solution or 10% formalin in sea water for later detailed observations of sectioned material. For microscopic examination of the skeletal system larvae were fixed in 70% alcohol, then macerated in a 10% aqueous solution of potassium hydroxide.

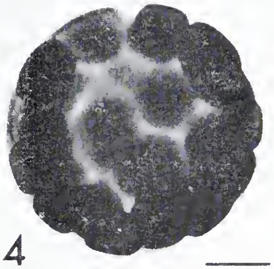
OBSERVATIONS

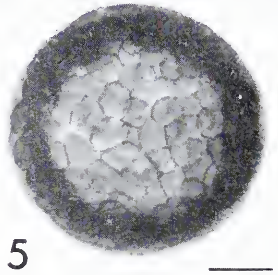
The mature ova are spherical, semitranslucent, brownish cream in color, and are enclosed in a jelly layer about 10 μ thick. They measure from 250 μ to 400 μ in diameter with a mean of about 300 μ. They are heavier than sea water.

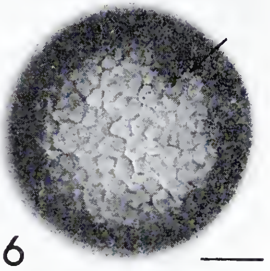


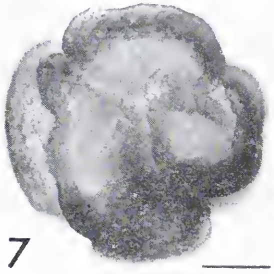


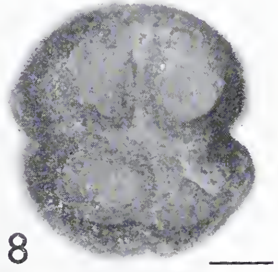












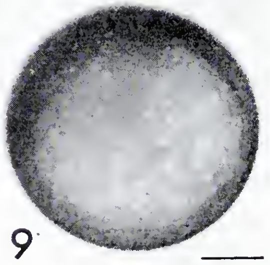


FIGURE 1. Fertilized egg with elevated fertilization membrane; scale = 100 μ.

FIGURE 2. Two-cell stage; scale = 100 μ.

FIGURE 3. Eight-cell stage; scale = 100 μ.

FIGURE 4. Thirty two-cell stage; scale = 100 μ.

FIGURE 5. Early coeloblastula; scale = 100 μ.

FIGURE 6. Beginning of wrinkled blastula stage. Arrow points egression tract; scale = 100 μ.

FIGURE 7. Wrinkled blastula with conspicuous egression tracts, 2 hours later than Figure 6; scale = 100 μ.

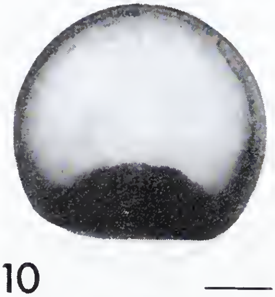
FIGURE 8. Later wrinkled blastula stage; scale = 100 μ.

FIGURE 9. Blastula just after resuming smooth surface; scale = 100 μ.

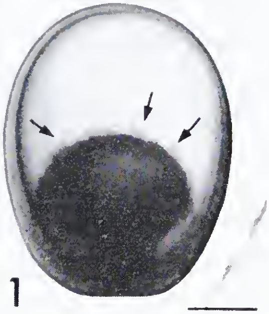
About two or three minutes after insemination, the fertilization membrane began to elevate, and 60 minutes thereafter the process was completed, with a perivitelline space 50 μ height (Fig. 1). The cleavage is of holoblastic, radial type. Seventy minutes after insemination, the first cleavage occurred through the animal-vegetal axis (Fig. 2). The embryos were in the 8-cell stage 120 minutes after insemination (Fig. 3), and in the 32-cell stage 150 minutes after insemination (Fig. 4). They developed into early coeloblastulae 3.5 hours after insemination (Fig. 5). Four hours after insemination, streaks (egression tracts) appeared on the surface of the blastula and then the embryos were entering the wrinkled blastula stage (Fig. 6). The egression tracts gradually increased in number and size, and embryos reached the most wrinkled stage 2 hours after the beginning of wrinkling (or 6 hours after insemination; see Fig. 7). Duration and magnitude of wrinkling, however, seemed to be somewhat different among individuals. Then, the egression tracts began to decrease in number and complexity (Fig. 8). Seven and one half hours after insemination, the surface of the blastulae resumed smoothness (Fig. 9). Eight hours after insemination gastrulation took place at the vegetal pole (Fig. 10). As far as can be ascertained from the present observations, the invagination for gastrulation apparently had no connection with egression tracts for wrinkling. Nine hours after insemination, early gastrulae began to rotate within the fertilization membrane.

One hour thereafter (10 hours after insemination) they hatched as free-swimming larvae. Two or three hours after hatching (12–13 hours after insemination), the blind end of the archenteron expanded, and then mesenchymal cells were set free into the blastocoel (Fig. 11). Ten hours after hatching (20 hours after insemination), a pair of the rudimental coelomic pouches was recognized at both sides of the tip of the archenteron. The gastrulae gradually elongated along the archenteric axis, and by 20 hours after insemination they had reached a length of 600 μ (Fig. 12). A few hours later, the archenteron seemed to be differentiated into stomach and intestine (Fig. 13). However, the details of the internal organogenesis could not be observed from outside due to the opacity of the larvae.

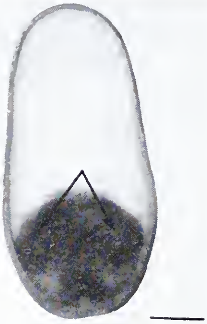
Thirty hours after insemination, the larvae showed a barrel form as shown in Figure 14. The length and the width of larvae at this stage were 700 μ and 350 μ, respectively, and the coelomic pouches were separated from the archenteron. The posterior wall of the larva was thicker than the anterior wall. Then the posterior end began to become rounded. Forty-five hours after insemination, 5 lobes of the hydrocoel became evident (Fig. 15). The posterior part of a larva corresponds to the future body of the sea-star after metamorphosis, and might be called a larval disk. On the other hand the anterior part, which will be reduced in size during metamorphosis, corresponds to the stalk of the brachiolaria larva. Two or three hours thereafter (47–48 hours after insemination), the distinction of these parts became more evident (Fig. 16). At that time both coelomic pouches extended so as to contact with each other by their anterior ends. Fifty hours after insemination, the skeletal plates began to be formed as spicules on the future aboral side of the larval disk (Fig. 28a). These spicules correspond to 1 madreporic and 5 terminal plates (Fig. 28b). Fifty-five hours after insemination, 10 processes arose on the peripheral part of the larval disk (Fig. 17). The hydrocoel was recognized in the future oral side of the larval disk (Fig. 18). At the opposite side of the larval disk, a central plate appeared as a spicule (Figs. 19 and 20). There were several spines on the terminal plate. A little while after, 5 pairs of rudimental oral plates appeared on the future oral side of the larval disk (Fig. 21). About 70 hours after insemination, the larvae moved slowly while the top of their future aboral side was in contact with the surface of the substratum (Fig. 22). On the fourth day of development (about 75 hours after insemination),



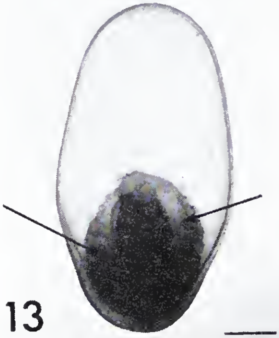
10



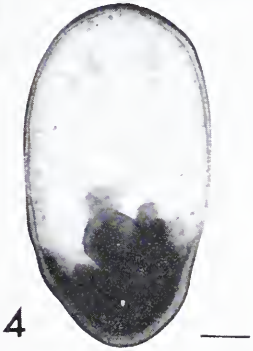
11



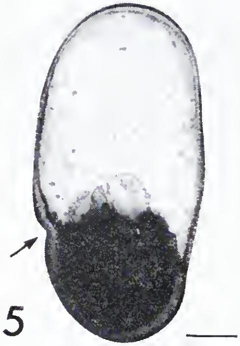
12



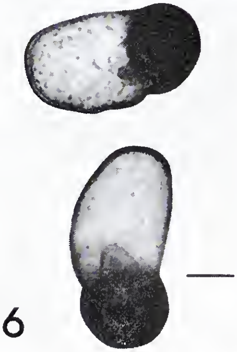
13



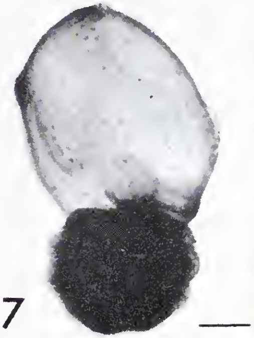
14



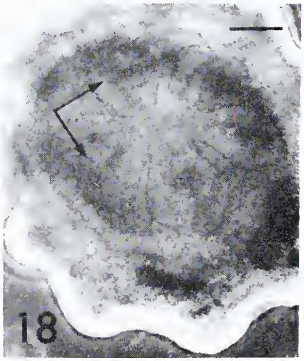
15



16



17



18

FIGURE 10. Early gastrula, just after beginning of invagination; scale = 100 μ.

FIGURE 11. Gastrula little later than that shown in Figure 10. Note expanded top of archenteron and liberation of mesenchymal cells (arrows); scale = 100 μ.

FIGURE 12. Gastrula, 20 hours after insemination. Arrows show rudiments of coelomic pouch; scale = 100 μ.

FIGURE 13. Gastrula; 5 hours later than that shown in Figure 12. Each coelomic pouch (arrows) elongates near the posterior end of larva; scale = 100 μ.

FIGURE 14. Larva in a barrel form, lateral view. Each coelomic pouch is separated from the archenteron; scale = 100 μ.

FIGURE 15. Metamorphosing larva, 45 hours after insemination. Note constriction (arrow) between disk and stalk; scale = 100 μ.

FIGURE 16. Two larvae showing opposite side to each other, one (upper) shows future aboral side and the other future oral side; 2 or 3 hours later than that shown in Figure 15, scale = 200 μ.

larvae attached to the substratum with the anterior portions of their stalks (Fig. 23).

Metamorphosis proceeded rapidly after attachment. The stalk began to degenerate (Fig. 24). The rudiments of two pairs of tube-feet and of a single terminal tentacle appeared on each lobe of the hydrocoel. At the same time, the formation of the skeletal system progressed further. Several spicules which correspond to the radial and interradial plates appeared around the central plate on the future aboral side (Figs. 30a and 30b). On the reverse side of the disk, the rudimental ambulacral plates appeared (Figs. 31a and 31b). The juvenile shown in Figure 25 was photographed 20 hours after attachment (or about 95 hours after insemination). The reduced stalk still remained in this stage. About 5 days after insemination the stalk was completely absorbed, and the opening of the mouth was recognized at the center of the oral side. Juveniles immediately after metamorphosis were white in color and 500 μ in diameter (Fig. 26). They had 2 long spines, each 10 μ in length, at the top of each of 5 arms. The skeletal system in this stage is shown in Figures 32a, 32b and 32c. Later an eye-spot appeared on the basal portion of each terminal tentacle (Fig. 33). At this stage, the tube-feet were functional having suckers at their tips, enabling juveniles to move about. Twenty-five days after insemination, each arm was distinguishable from the disk. At that time, a central plate and other dorsal plates developed as shown in Figure 27.

Although about 300 juveniles were alive in the laboratory for about 40 days after insemination, they did not show further differentiation and eventually died. *Artemia*-larvae or clam meat were not acceptable as food. The skeletal system of the juvenile, which is 300 μ in R (the distance from the center of the disk to the tip of the arm) and 200 μ in r (the distance from the center of the disk to the middle of the interradial margin), is given in Figures 34a, 34b and 34c. As shown in the figures, each arm has a terminal plate with about 10 spines. Dorsal plates, with 1 spine at the center, lie compactly on the aboral side of the disk. A madreporic plate was recognizable on one interradius. Five pairs of oral and ambulacral plates developed on the oral side.

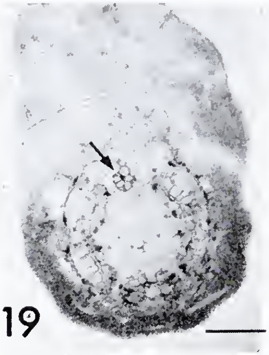
Discussion

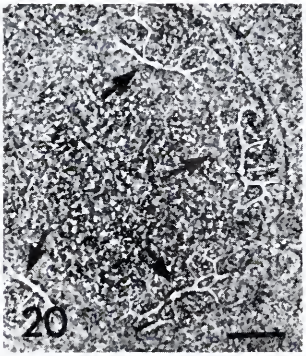
In the present study, it was found that the development of *Astropecten latespinosus* is quite different from that of the other sea-stars, in the following three points: (1) there is a peculiar larval form, (2) metamorphosis takes place while the larva is pelagic; there is no brachiolaria stage; (3) the larval life is very short.

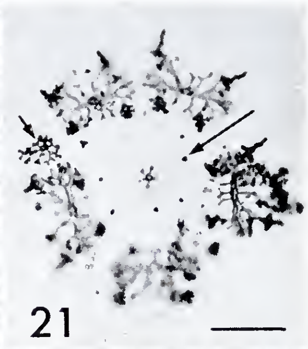
There has been no previous report of the swimming larvae of asteroids having the morphology described here. They are barrel-shaped, somewhat resembling the doliolaria larva but lacking in definite ciliary bands. Metamorphosis is initiated at the posterior part of the body while the larva is pelagic; fixing disk and brachiolar arms are not formed, although these are usually found during metamorphosis in sea-stars. The larva completes metamorphosis without feeding, as reported

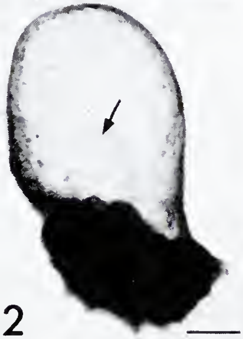
FIGURE 17. Larva, 55 hours after insemination; scale = 100 μ.

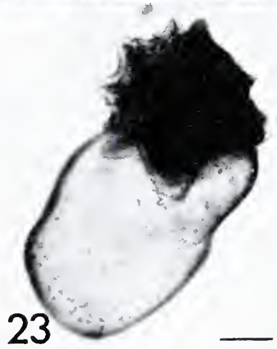
FIGURE 18. Larval disk showing future oral side. Same stage as shown in Figure 17. Arrows point primordial lobes of hydrocoel; scale = 50 μ.

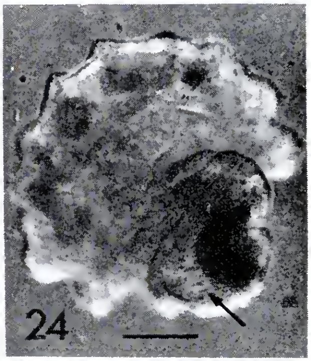


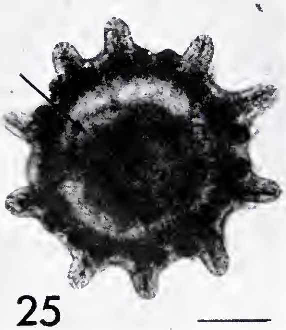


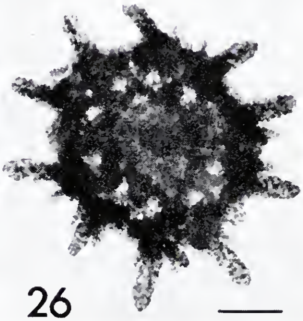












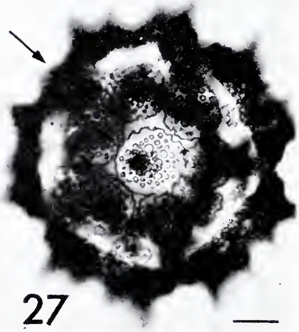


FIGURE 19. Opposite side (future aboral side) of the same larva as in Figure 18, showing rudimental skeletal plates. Arrow indicates madreporic plate; scale = 100 μ.

FIGURE 20. Enlarged picture of a part of the disk shown in Figure 19, showing details of terminal plates indicated by arrows (phase contrast microscopy); scale = 50 μ.

FIGURE 21. Skeletal system of the larva, slightly later than that shown in Figure 19; prepared by the treatment of KOH solution. Short and long arrows point to madreporic plate and oral plate, respectively; scale = 100 μ.

FIGURE 22. Larva just before attachment, lateral view. Arrow shows the anterior coelom; scale = 100 μ.

FIGURE 23. Four day-old larva, just after attachment to the substratum; scale = 100 μ.

FIGURE 24. Attached larva, future oral side, pictured through the glass bottom. Arrow points degenerating stalk; scale = 100 μ.

for some species such as *Crossaster papposus, Leptasterias ochotensis similispinis* or *Ceratonardoa semiregularis* (Gemmill, 1920; Kubo, 1951; Hayashi and Komatsu, 1971).

Development of the sea-stars has been generally divided into two types, the indirect and the direct, mainly based on what type of larva appears. In indirect development, the embryo develops into a brachiolaria after passing through the bipinnaria stage; it attaches to the substratum by fixing disk or brachiolar arms before the completion of metamorphosis. This type of development has been reported in *Asterias rubens, Porania pulvillus, Asterias amurensis* and *Acanthaster planci* (Gemmill, 1914, 1915; Dan, 1957; Yamaguchi, 1971). The second type, direct development, passes through only the brachiolaria stage; a bipinnaria stage is entirely lacking. *Asterina gibbosa, Henricia sanguinolenta, Solaster endeca, Echinaster echinophorus* are among those having direct development (MacBride, 1896; Masterman, 1902; Gemmill, 1912; Atwood, 1973). It may be an object of argument to classify the type of development of the present species, because it does not form either typical bipinnaria or brachiolaria. The distinction, however, between those two types has not given very clearly. An attempt has been made by some workers to give a precise definition for two types of development. Hyman (1955) described the direct type as a kind of development lacking in free larvae. However, one may regard the swimming brachiolariae found in *Crossaster papposus* or *Certonardoa semiregularis* as free larvae in spite of the fact that they undergo direct development. It was proposed by Chia (1968) that development with a larva having a functional larval gut should be called the indirect type and those without a functional gut the direct type. This distinction may be useful in dividing all asteroid larvae into two types. The development of *Astropecten latespinosus*, therefore, is of the direct type according to Chia's classification.

It appears that there is an intimate relationship between the size of the egg and the type of development. Eggs having direct development are generally larger than those with indirect development. The largest egg having indirect development, is that of *Astropecten scoparius* which is 230 μ in diameter (Komatsu, 1973). On the other hand, eggs characterized by direct development are more than twice the size of those with indirect development (see Hayashi, 1972); the smallest so far reported is about 500 μ in *Asterina gibbosa* (MacBride, 1896). The majority of the sea-stars undergoing direct development take more than 30 days to complete metamorphosis after fertilization, except *Echinaster echinophorus* (Atwood, 1973) which takes about 14 days to complete metamorphosis. The egg of the present species is about 300 μ in diameter, and is thus being very small among eggs with direct development. The present species completes metamorphosis within

FIGURE 25. More advanced stage than that shown in Figure 24, future oral side. Note the bulges for 2 pairs of tube-feet and ten long spines on the terminal plates. Arrow shows reduced stalk; scale = 100 μ.

FIGURE 26. Juvenile immediately after the completion of metamorphosis, oral view; scale = 100 μ.

FIGURE 27. Aboral skeletal plates of a juvenile, 25 days after insemination. Note large central plate in the center and radial and interradial plates around it. Upper left (arrow) shows madreporic plate. Prepared by the treatment of KOH solution; scale = 50 μ.

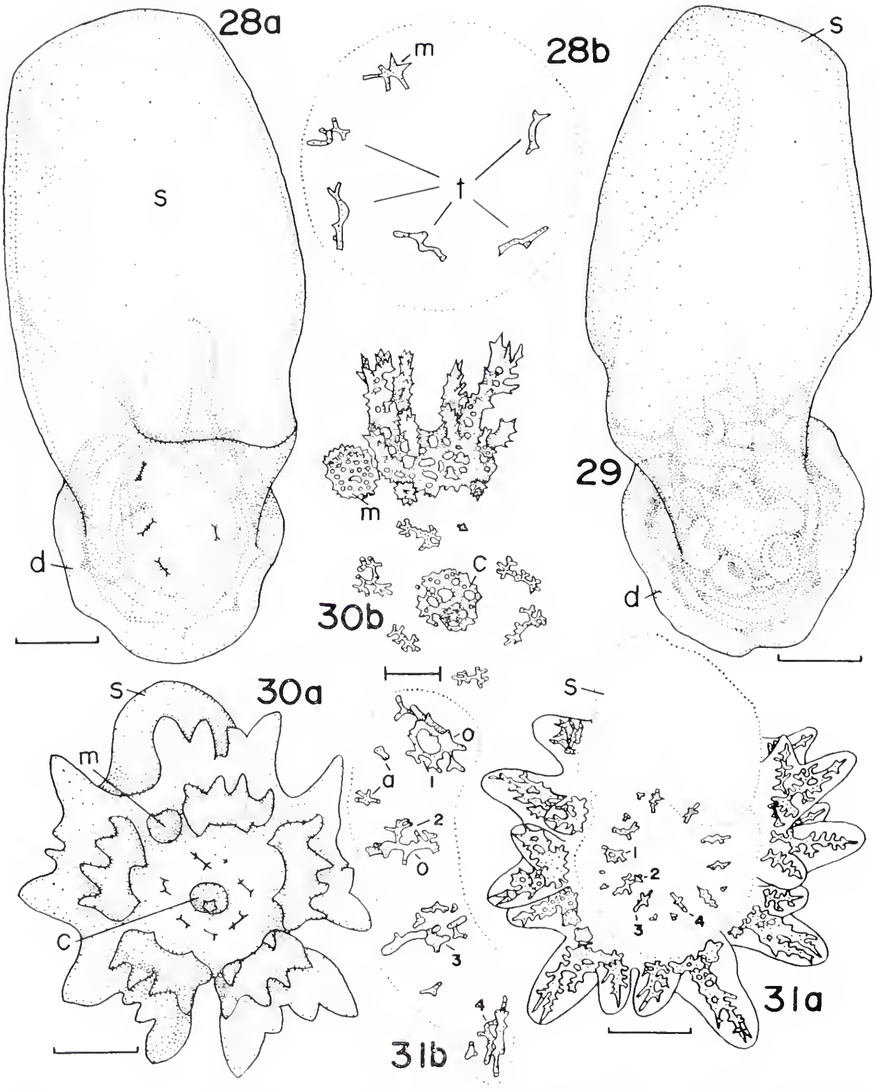


FIGURE 28a. Free-swimming larva, 50 hours after insemination, showing future aboral side of disk; d, disk; s, stalk; scale = 100 μ.

FIGURE 28b. Primordia of the skeletal plate, same stage as shown in Figure 28a; m, madreporic plate; t, terminal plate.

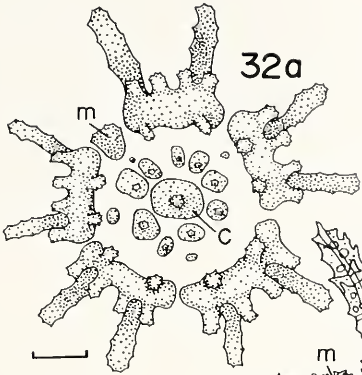
FIGURE 29. Opposite side (future oral side) of that shown in Figure 28a; d, disk; s, stalk; scale = 100 μ.

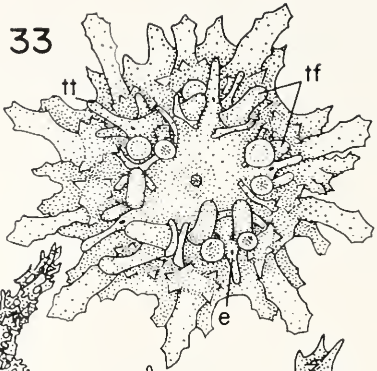
FIGURE 30a. Skeletal system of attached larva, future aboral side; c, central plate; m, madreporic plate; s, stalk; scale = 100 μ.

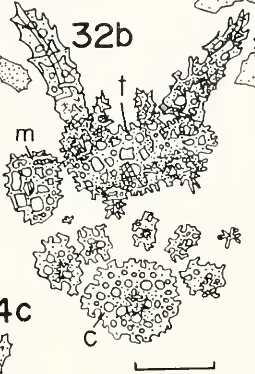
FIGURE 30b. Detailed sketch of plates, same specimen as shown in Figure 30a; c, central plate; m, madreporic plate; scale = 25 μ.

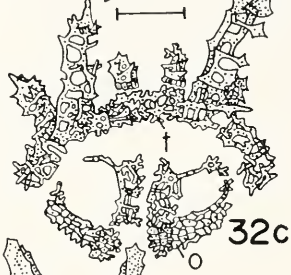
FIGURE 31a. Skeletal system, opposite side of that shown in Figure 30a; s, stalk; scale = 100 μ.

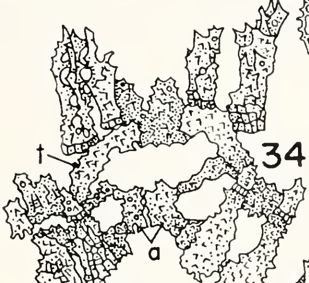
FIGURE 31b. Detailed sketch of oral and ambulacral plates, same specimen as shown in Figure 31a. Numbers (1, 2, 3 and 4) correspond to the numbers (1, 2, 3 and 4) of Figure 31a; a, ambulacral plate; o, oral plate; scale = 25 μ.

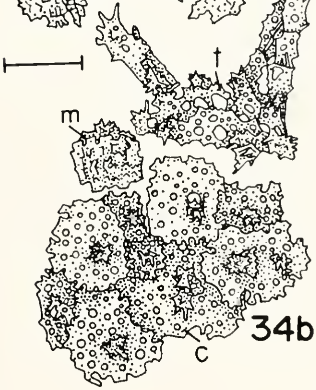












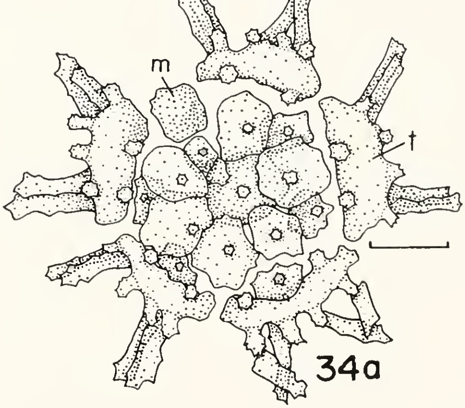


FIGURE 32a. Aboral skeletal system of the juvenile, same stage as shown in Figure 26; m, madreporic plate; c, central plate; scale = 100 μ.

FIGURE 32b. Detailed sketch of plates, same specimen as shown in Figure 32a; c, central plate; m, madreporic plate; t, terminal plate; scale = 75 μ.

FIGURE 32c. Detailed sketch of plates on oral side, same specimen as shown in Figure 32b; o, oral plate; t, terminal plate; scale = 50 μ.

FIGURE 33. Oral side of the juvenile, little later than that shown in Figure 26; e, eyespot; tf, tube-foot; tt, terminal tentacle; scale = 50 μ.

FIGURE 34a. Aboral skeletal system of 40 day-old juvenile; m, madreporic plate; t, terminal plate; scale = 100 μ.

FIGURE 34b. Detailed sketch of plates, same specimen as shown in Figure 34a; c, central plate; m, madreporic plate; t, terminal plate; scale = 75 μ.

FIGURE 34c. Detailed sketch of plates, oral side of the same specimen as shown in Figure 34; a, ambulacral plate; t, terminal plate; scale = 75 μ.

7 days after insemination and this speedy development may be related to the small size of the egg.

It was reported for all species of the genus *Astropecten* thus so far studied that metamorphosis takes place while larvae are pelagic as typical bipinnaria (Mortensen, 1921, 1937; Hörstadius, 1939). As noted above, *Astropecten latespinosus* also undergoes metamorphosis while the larvae are pelagic, although it does not form bipinnaria, but rather a barrel-shaped larva. The present species thus may show a much abbreviated process of development in comparison with the other species of *Astropecten*; presumably this is due to the lecithotrophic nature of the egg.

The writer wishes to express her cordial thanks to Professor Emeritus Ryoji Hayashi and Professor Chitaru Oguro, Toyama University, for their unfailing guidance. Her gratitude is also due to Professor Emeritus Katsuma Dan, Tokyo Metropolitan University, for his interest in the present study and revision of the manuscript for publication.

SUMMARY

1. The entire process of development in the sea-star, *Astropecten latespinosus*, is reported, especially with regard here to the external morphology and the skeletal system.

2. The eggs are medium-sized, about 300 μ in diameter. They develop into free-swimming larvae through a wrinkled blastula stage by holoblastic, radial cleavage.

3. The free-swimming larva has a peculiar barrel shape, being neither bipinnaria nor brachiolaria. Such a larva has not previously been reported.

4. Metamorphosis takes place while the larva is pelagic, and there is no feeding at this stage. Five days after insemination, metamorphosis is completed and the resulting juveniles bear 2 pairs of tube-feet and a terminal tentacle in each arm.

5. The present observations are compared with those studied by other workers, and are discussed, with special reference to the type of development of sea-stars.

LITERATURE CITED

ATWOOD, D. G., 1973. Larval development in the asteroid *Echinaster echinophorus*. *Biol. Bull.*, **144**: 1–11.

CHIA, F-S., 1968. The embryology of a brooding starfish, *Leptasterias hexactis* (Stimpson). *Acta Zool.*, **49**: 321–364.

DAN, K., 1957. IX-3 Asteroidea. Pages 215–218 in M. Kume and K. Dan, Eds., *Invertebrate Embryology*. Baifukan, Tokyo. (In Japanese)

GEMMILL, J. F., 1912. The development of the starfish *Solaster endeca* Forbes. *Trans. Zool. Soc. London*, **20**: 1–71.

GEMMILL, J. F., 1914. The development and certain points in the adult structure of the starfish *Asterias rubens*, L. *Phil. Trans. Roy. Soc. London*, **205**: 213–294.

GEMMILL, J. F., 1915. The larva of the starfish *Porania pulvillus* (Q. F. M.). *Quart. J. Microscop. Sci.*, **61**: 27–50.

GEMMILL, J. F., 1920. The development of the starfish *Crossaster papposus*, Müller and Troschel. *Quart. J. Microscop. Sci.*, **64**: 155–189.

HAYASHI, R., AND M. KOMATSU, 1971. On the development of the sea-star, *Certonardoa semiregularis* (Müller et Troschel) I. *Proc. Jap. Soc. Syst. Zool.*, **7**: 74–80.

HAYASHI, R., 1972. On the relations between the breeding habits and larval forms in asteroids, with remarks on the wrinkled blastula. *Proc. Jap. Soc. Syst. Zool.*, **8**: 42–48.

HÖRSTADIUS, S., 1939. Über die Entwicklung von *Astropecten aranciacus* L. *Pubbl. Staz. Zool. Napoli*, **17**: 221–312.

HYMAN, L. H., 1955. *The Invertebrates, IV Echinodermata.* McGraw-Hill, New York, 763 pp.

KANATANI, H., 1969. Induction of spawning and oocyte maturation by 1-methyladenine in starfishes. *Exp. Cell Res.*, **57**: 333–337.

KOMATSU, M., 1973. On the wrinkled blastula of the sea-star, *Astropecten scoparius. Zool. Mag.*, **82**: 204–207. (In Japanese with English abstract)

KUBO, K., 1951. Some observations on the development of the sea-star, *Leptasterias ochotensis similispinis* (Clark). *J. Fac. Sci., Hokkaido Univ.*, **10**: 97–105.

MACBRIDE, E. W., 1896. The development of *Asterina gibbosa. Quart. J. Microscop. Sci.*, **38**: 339–411.

MASTERMAN, A. T., 1902. The early development of *Cribrella oculata* (Forbes), with remarks on echinoderm development. *Trans. Roy. Soc. Edinburgh*, **40**: 373–418.

MORTENSEN, TH., 1921. *Studies of the Development and Larval Forms of Echinoderms.* G. E. C. Gad., Copenhagen, 261 pp.

MORTENSEN, TH., 1937. Contributions to the study of the development and larval forms of echinoderms. III. *Kgl. Dan. Vidensk. Selsk, Skr. Naturvid. Math. Afd., Series 9*, **7**(1): 1–44.

NEWTH, H. G., 1925. The early development of *Astropecten irregularis*, with remarks on duplicity in echinoderm larvae. *Quart. J. Microscop. Sci.*, **69**: 519–554.

YAMAGUCHI, M., 1971. Larval development and metamorphosis of *Acanthaster planci* (Asteroidea). *Nature*, **232**: 655–657.

Reference: *Biol. Bull.*, **148**: 60–67. (February, 1975)

A RADIOACTIVE TRACER STUDY OF FOOD UPTAKE BY *PINNOTHERES MACULATUS* IN MOLLUSCAN HOSTS

W. L. KRUCZYNSKI

Saline Marsh Ecology Project, Florida A & M University, Tallahassee, Florida 32307

The Pinnotheridae, pea crabs, is a group of decapod crustaceans adapted for living with other marine animals. Pearce (1962) discussed adaptations of these crabs for symbiotic existence, and Gotto (1969) listed bivalves, gastropods, sea slugs, chitons, polychaetes, echinoderms, burrowing crustaceans and sea squirts as known hosts.

Associations of most pinnotherids with their hosts have not been defined. Many have long been recognized as commensals, and some have been labeled parasitic (Cheng, 1967; Gotto, 1969). *Pinnotheres ostreum* (Say), for example, is known to cause gill and palp damage to *Crassostrea virginica* (Gmelin) and reduce growth and reproduction of the oyster (Overcash, 1946; Sandoz and Hopkins, 1947; Flower and McDermott, 1952; Haven, 1958; Christensen and McDermott, 1958). McDermott (1962) found that both *P. ostreum* and *Pinnotheres maculatus* Say caused similar damage in *Mytilus edulis* Linné. *Fabia subquadrata* Dana was observed to damage gills and cause cyst-like anomalies in mantle tissues of *Modiolus modiolus* Linné (Pearce, 1966a) and *Pinnotheres* sp. damaged gill, mantle and gonad of *Meretrix casta* (Chemnitz) (Silas and Alagarswami, 1967).

The mode of feeding of some pinnotherids has been investigated. Pearce (1966b) noticed that *Pinnixa fabra* (Dana) caused damage to its host clam, *Tresus capax* (Gould), and found that the crab ingested food which would otherwise be available to the clam. Stealing of food which a host has concentrated into food strings has also been reported for *Pinnotheres pisum* (Pennant) (Coupin, 1894; Orton, 1921), *Pinnotheres concharum* (MacGinitie and MacGinitie, 1968) and *P. ostreum* (Stauber, 1945). Campbell (1969) found plant pigments in the gut of *P. pisum* which indicated ingestion of algae. Orton (1921) and Gotto (1969) suggested that some crabs may also filter food from water currents created by hosts. All of these studies are descriptive and no experimental quantification of crab-host associations are known except that adult female *P. maculatus* stunted growth of bay scallops, *Argopecten irradians concentricus* (Say), grown in cages (Kruczynski, 1972).

Radioisotope techniques are common tools to study physiological processes, but few studies are known employing these methods to quantify trophic relationships in natural ecosystems. Poole (1974) discussed some methods of measuring ingestion using radioactive tracers and reviewed research which quantified components of some terrestrial and stream ecosystems by these methods. Smith, Muscatine and Lewis (1969) reviewed quantification of carbohydrate movement using ^{14}C from autotrophs to heterotrophs in parasitic and mutualistic symbiosis. Pardy and Muscatine (1973) used labeled green algae to quantify uptake of symbionts by *Hydra viridis*.

Phytoplankton labeled with radioisotopes has been used to measure water filtering rates of some ascidians and lamellibranchs (Jorgensen and Goldberg, 1953; Chipman and Hopkins, 1954; Rice and Smith, 1958). This present study reports use of phytoplankton labeled with ^{14}C to quantify *in situ* the association of adult female *P. maculatus* with its common hosts, the bay scallop, *A. irradians*, calico scallop, *Argopecten gibbus* (Linné), and blue mussel, *M. edulis*, under laboratory conditions.

Materials and Methods

Collection and maintenance of organisms

Feeding experiments with bay and calico scallops were conducted in North Carolina. Bay scallops were collected from Bogue Sound and calico scallops from off Cape Lookout, North Carolina. Scallops were cleaned of encrusting organisms and kept at room temperature (20–25° C) in large plastic trays containing aerated seawater. Feeding experiments with blue mussels were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts. Mussels were collected from pier pilings of the Woods Hole Oceanographic Institution and kept in running seawater at room temperature (20–22° C).

Preliminary observations insured that selected diatoms were filtered and ingested by mollusc hosts. *Nitzchia closterium* was used in experiments to follow uptake of phytoplankton into scallops, crabs in scallops and crabs in finger bowls containing phytoplankton. *Thalassiosira pseudonana* was used in experiments with mussels. Cultures were grown at room temperature in half-strength Guillard's medium (Guillard and Ryther, 1962) prepared with 30‰ seawater. Radioactive carbon was added as $NaH^{14}CO_3$ before inoculation of new medium with stock culture, and cultures grew at least 7 days before used in an experiment. Assay revealed that much of the tag was found in the diatoms, although some was left in solution.

Assay of radioactivity

Radioactivity in scallop experiments was determined with a Dynacon (Nuclear Chicago, Model 6010). This instrument has been used in studying carbon fixation by aquatic plants (Dillon, 1971) and is sensitive to low amounts of β radiation. It has a counting efficiency of nearly 100% but only a few samples can be run per day. Radioactivity of CO_2 in dry, combusted samples is measured. A Packard Liquid Scintillation Spectrometer was used in mussel experiments. Samples were oxidized in a Packard Tri-Carb Automatic Sample Oxidizer. Resultant $^{14}CO_2$ was absorbed in scintillation vials. Quenching characteristics of oxidized samples are nearly constant (Faires and Parks, 1973) and no quench corrections were made. Counting efficiency of this system was nearly 80%.

Bay scallops

Background radioactivity of dried tissues of bay scallops was determined by assaying aliquots. Uptake of ^{14}C from solution was measured by filling two bowls with 1 liter of seawater and 1.6 μCi ^{14}C (2.2×10^6 cpm/μCi). A bay scallop was placed in each bowl for 24 hours and water was aerated with breakerstones. On removal, scallops were rinsed with filtered water and dried to a constant weight at 80° C. Aliquots of scallop tissues were assayed and total radioactivity estimated.

Feeding experiments were performed with six noninfected scallops. A control bowl (no scallop) was run per pair of experimental bowls. Bowls were filled with 1 liter of water and a measured amount of radioactive culture of *N. closterium*. Cell counts were made to assure approximate equality and bowls were covered with a glass plate and aerated. A scallop was placed in each experimental bowl for 24 hours, after which contents of control and experimental bowls were centrifuged in a continuous plankton centrifuge and filtered with a Millipore filter (HA 0.45 μ). Particulate matter from the control bowl was used as an estimate of initial phytoplankton activity; that from experimental bowls represented phytoplankton that was rejected as faeces, pseudofaeces and unfiltered phytoplankton. No effort was made to separate activity in the three categories of "rejected materials."

Experimental scallops were then placed in filtered water for 24 hours, removed from the shells, dried and weighed. Contents of bowls containing scallops for the second day were recovered and the activity added to the rejected material. Aliquots of scallops and whole dried filteres were assayed and total counts per minute (cpm) per scallop estimated.

Twelve additional bay scallops were infected with an adult female crab 3 days prior to experiments. I amputated the chelae from one crab before placing it in a scallop. Crabs were kept isolated in finger bowls for at least 7 days before being placed in scallops.

A control bowl was run per pair of scallops tested and bowls were treated as above. After the second day, crabs and scallop tissues were dried, weighed and assayed.

Calico scallops

Two calico scallops, each containing its naturally occurring adult female crab, were tested as above. A control bowl was run for each.

Blue mussels

Seven mussels were selected from an area where most were known to contain an adult female crab. They were placed in bowls containing a known concentration and activity of radioactive *T. pseudonana* and allowed to feed for 24 hours. Mussels were then placed in filtered seawater for 24 hours. In one trial the chelae of an adult female crab were removed and it was placed back into a mussel which was relaxed in isosmotic $MgCl_2$ and kept in running seawater for one day before testing. One control mussel and crab was treated in the same manner except no radioactive phytoplankton was added to the dish.

Pinnotheres maculatus *in finger bowls*

Six crabs were tested to determine whether crabs can ingest phytoplankton which has not been concentrated by a host. Each trial included two experimental and one control bowl. The same amount of a culture of tagged specimens of *N.*

TABLE I

Uptake of radioactivity by adult female specimens of Pinnotheres maculatus *and molluscan hosts. Mean values given, standard deviations in parentheses.*

Species	n	Initial diatoms cells/ml × 10^3	Host cpm/mg	Crab cpm/mg	Per cent of initial radioactivity			
					Recovered	In host	In crab	Rejected
A. irradians								
No crabs	6	210	151(40)	—	81(24)	23(14)	—	58(30)
With crab	11	240	188(108)	195(130)	78(10)	40(11)	3(1)	36(19)
With clawless crab	1	810	87	0	97	35	0	62
A. gibbus								
With crab	2	150	419(7)	823(58)	85(11)	40(11)	7(2)	38(3)
M. edulis								
No crabs	2	330	16(6)	—	95(30)	24(5)	—	71(37)
With crab	5	340	18(5)	142(146)	78(9)	28(12)	2(1)	48(8)
With clawless crab	1	350	15	27	75	6	2	67
Finger bowl								
Clawed crab	5	144	—	171(130)	79(11)	—	21(8)	58(10)
Clawless crab	1	110	—	15	86	—	3	83

closterium was added to the bowls in each trial. A female crab was placed in each of the experimental bowls which were shaded with paper towels. After 24 hours, crabs were rinsed with filtered water, blotted dry, and contents of all bowls filtered. One experimental bowl contained a crab with amputated chelae.

RESULTS

Bay scallops

Background activity of bay scallops was 0.20 cpm/mg dry weight. Scallops kept in water with ^{14}C in solution became tagged and contained an estimated 3500 cpm (3 cpm/mg) probably because of physical exchange of water, incomplete washing of tissues, or both. This uptake may be a source of error in other experiments, but it was disregarded as a major factor because of the low activity for the amount of ^{14}C used.

Since the amount of radioactivity per amount of phytoplankton varied between trials in feeding experiments, comparison of actual numbers between experiments are less valuable than comparing percent recovery of initial tagged material.

In experiments with noninfected bay scallops, recovery of radioactivity varied from 42 to 100% of estimated initial activity (Table I). Total radioactivity of noninfected scallops varied from 9 to 41% and rejected material, that is faeces, pseudofaeces and uneaten diatoms, varied from 19 to 88% of the initial tag. There was much pseudofaeces in all bowls containing scallops. All scallops were radioactive and activity varied from 90 to 206 cpm/mg, indicating an accumulation of ^{14}C under these conditions. There was little correlation between scallop dry weight and per cent uptake ($r = 0.45$).

In experiments with bay scallops infected with crabs, recovery of initial radioactivity varied from 66 to 97%. Scallops and crabs rejected 12 to 72% and scallops contained 16 to 53% of the estimated initial tag. Radioactivity of scallops varied from 51 to 337 cpm/mg. Crabs contained between 1 and 6% of the initial tag and activity varied from 64 to 396 cpm/mg (Table I), indicating that clawed crabs accumulate ^{14}C under these conditions. There was little correlation between crab dry weight and percent uptake ($r = 0.39$). The clawless crab contained no radioactivity above background.

Calico scallops

Calico scallops became tagged with 414 and 424 cpm/mg dry weight. Crabs contained 6 and 9% of initial tag and had an activity of 782 and 864 cpm/mg.

Blue mussels

Control mussel and crab contained no radioactivity above background. Between 65 and 118% of initial radioactivity was recovered. Mussels contained from 6 to 44% of the initial radioactivity on diatoms and had an activity of between 5 and 24 cpm/mg. There was little correlation between mussel weight and per cent uptake ($r = 0.46$). Five of the mussels harbored a female crab and crabs contained 24 to 355 cpm/mg. The clawless crab accumulated 27 cpm/mg. The correlation coefficient of crab uptake and dry weight was 0.23.

Pinnotheres maculatus *in finger bowls*

Clawed crabs became tagged with 59 to 396 cpm/mg. The clawless crab contained 15 cpm/mg (Table I). Between 7 and 28% of initial activity added as phytoplankton was found in clawed crabs. Gut analysis confirmed that crabs ingested diatoms.

Discussion

The morphology of adult female specimens of *P. maculatus* is specialized for existence in mollusc hosts. Males are specialized for swarming and are capable of living outside hosts. Patton (1967) observed that commensal crustaceans found in sheltered situations have a stout form, often accompanied by increased egg production, and a soft exoskeleton which could be selectively favored by not irritating hosts enough to seriously disturb food intake. I have placed adult female specimens of *P. maculatus* into noninfected bay scallops and have observed that three to five days is ample time for hosts to adapt to the presence of crabs and resume normal filtration rates (Kruczynski, 1971).

Pearce (1962) also noted considerable differences in the integument of pinnotherids and the remainder of brachyurans. Males generally have a hard exoskeleton and their importance seems restricted to copulation. Patton (1967) suggested that any mechanism which would result in noncommensal males would have a selective advantage because in some cases a single female crab can seriously affect a host. Thus, males were not used in my study because their effect on nutrition of a single host is probably small since they move from host to host.

This should be tested in future experiments. Adult female crabs, however, are virtually trapped in the mantle cavity of molluscs and have more effect on a single host. Demonstrating that female specimens of *P. maculatus* accumulate ^{14}C in scallops and mussels feeding on labeled diatoms strengthens the argument for its being the causative agent of observed stunting of bay scallops under natural and experimental conditions (Kruczynski, 1972).

Many commensal crabs rely on hosts to concentrate food into food strings. My results indicate that *P. maculatus* can also ingest *N. closterium* which has settled in finger bowls. These crabs were observed to actively pick at the bottom of the dishes and continuously clean themselves. It is still not known whether *P. maculatus* has any ability to filter their own food. Adults seem unadapted morphologically for that mode of feeding. The fact that clawless crabs in scallop, mussel and dish had low activities supports this.

Bivalves filter large volumes of water in feeding. Chipman and Hopkins (1954) followed the decrease of radioactive *N. closterium* from the medium to demonstrate that bay scallops (64 mm shell height) filtered about 15 liters per hour. They found no apparent difference in filtering rate in suspensions of phytoplankton of different concentrations and they did not measure how much phytoplankton was ingested. Rice and Smith (1958) found that *Mercenaria mercenaria* (Linné) filtered *N. closterium* in concentrations between 400 cells/ml to 170×10^3 cells/ml. Clams formed large amounts of pseudofaeces in high concentrations of algae. Perhaps the large percentage of tag found in pseudofaeces (rejected material) in my experiments is the result of using high cell concentrations. Further studies might be more useful if natural concentrations of phytoplankton were used. It is probable that effects of removal of phytoplankton by crabs from hosts is more pronounced under conditions of natural phytoplankton concentrations.

Experimental *P. maculatus* accumulated 3%, 7% and 2% of radioactivity used in bay scallop, calico scallop and mussel experiments respectively, but it is still not known what part of this activity came from ingestion of phytoplankton, faeces, pseudofaeces, molluscan tissues, or from the water. Many workers have shown that some aquatic organisms take up more radionuclides from water than from food pathways (Mauchline and Templeton, 1964; Polikarpov, 1966). This error could be reduced in future experiments by washing phytoplankton cells before adding them to experimental dishes. Washing would also reduce error resulting from continued fixation of ^{14}C by diatoms while experiments were in progress. I think that direct uptake or physical sticking of phytoplankton cells to experimental animals were not important sources of error in my experiments because of the low correlations between dry weights and percent of tag recovered. If contamination was a major factor, there would be a direct relationship between the amount of tissue and activity of experimental animals in which nearly the same amount of tag was used. This was not observed.

Other pinnotherids should be examined in a similar way to determine food uptake in hosts so that associations may be defined. Tagged hosts might be used to determine ingestion of mucus or host tissues.

I thank Austin B. Williams, U. S. National Museum, for his guidance, William J. Woods, University of North Carolina Institute of Marine Sciences, for his help and use of laboratory space and equipment, and Frank Loewus, University of Buffalo and the Marine Biological Laboratory, Woods Hole, Massachusetts, for use of combustion and scintillation equipment obtained with NSF Grant GB 37149X. This study was supported by a Duke University Oceanographic Traineeship Award and a Hartwick College Research Grant.

SUMMARY

Adult, female specimens of *Pinnotheres maculatus* accumulated radioactivity when in bay and calico scallops fed *Nitzchia closterium* labeled with ^{14}C and in blue mussels fed labeled *Thalassiosira pseudonana*. Crabs accumulated radioactivity when kept in fingerbowls with labeled *N. closterium*.

LITERATURE CITED

CAMPBELL, S. A., 1969. Carotenoid metabolism in the commensal crab *Pinnotheres pisum*. *Comp. Biochem. Physiol.*, **30**: 803–812.

CHENG, T. C., 1967. Marine molluscs as hosts for symbiosis. *Advan. Mar. Biol.,* **5**: 1–424.

CHIPMAN, W. A., AND J. G. HOPKINS, 1954. Water filtration by the bay scallop, *Pecten irradians,* as observed with the use of radioactive plankton. *Biol. Bull.,* **107**: 80–91.

CHRISTENSEN, A. M., AND J. J. MCDERMOTT, 1958. Life history and biology of the oyster crab, *Pinnotheres ostreum* Say. *Biol. Bull.*, **114**: 146–179.

COUPIN, H., 1894. Sur l'alimentation de deux commensaux (*Nereilepas* et *pinnotheres*). *C.R. Acad. Sci. Paris*, **119**: 540–543.

DILLON, C. R., 1971. Comparative study of the primary productivity of the estuarine phytoplankton and macrobenthic plants in Bogue Sound, North Carolina. *Ph.D. dissertation. University of North Carolina*, Chapel Hill, 120 pp.

FAIRES, R. A., AND B. H. PARKS, 1973. *Radioisotope Laboratory Techniques.* Halsted Press Div., John Wiley and Sons, Inc., New York, 312 pp.

FLOWER, F. B., AND J. J. MCDERMOTT, 1952. Observations on the occurrence of the oyster crab, *Pinnotheres ostreum,* as related to the oyster damage in Delaware Bay. *Nat. Shellfish Ass., Conv. Addresses*, **1952**: 44–50.

GOTTO, R. V., 1969. *Marine Animals: Partnerships and Other Associations.* American Elsevier Publ. Co., Inc., 96 pp.

GUILLARD, R. R. L., AND J. H. RYTHER, 1962. Studies of marine plankton diatoms. I. *Cyclotella nana* Huredt, and *Detonula confervacea* (Cleve.) *Gran. Can. J. Microbiol.*, **8**: 229–239.

HAVEN, D., 1958. Effects of pea crabs, *Pinnotheres ostreum,* on oysters, *Crassostrea virginica. Proc. Nat. Shellfish Ass.*, **49**: 77–86.

JORGENSEN, C. BARKER, AND E. D. GOLDBERG, 1953. Particle filtration in some ascidians and lamellibranchs. *Biol. Bull.*, **105**: 477–489.

KRUCZYNSKI, W. L., 1971. Relationship of the pea crab, *Pinnotheres maculatus* (Say), with the scallops, *Argopecten irradians concentricus* (Say) and *Argopecten gibbus* (Linne). *Ph.D. dissertation, University of North Carolina*, Chapel Hill, 120 pp.

KRUCZYNSKI, W. L., 1972. The effect of the pea crab, *Pinnotheres maculatus* Say, on growth of the bay scallop, *Argopecten irradians concentricus* (Say). *Chesapeake Sci.,* **13**: 218–220.

MACGINITIE, G. E., AND N. MACGINITIE, 1968. *Natural History of Marine Animals.* McGraw-Hill, Inc., New York, 523 pp.

MAUCHLINE, J., AND W. L. TEMPLETON, 1964. Artificial and natural radioisotopes in the marine environment. *Oceanogr. Mar. Biol. Ann. Rev.,* **2**: 229–279.

MCDERMOTT, J. J., 1962. The incidence and host-parasite relations of pinnotherid crabs. *Proc. First National Coastal Shallow Water Res. Conf.,* **1962**: 162–164.

ORTON, J. H., 1921. The mode of feeding and sex phenomenon in the pea crab, *Pinnotheres pisum. Nature,* **106**: 533–534.

Overcash, A. E., 1946. The use of measurement to determine the condition of oysters in Virginia. *Masters thesis, College of William and Mary,* Williamsburg, 31 pp.

Pardy, R. L., and L. Muscatine, 1973. Recognition of symbiotic algae by *Hydra viridis.* A quantitative study of the uptake of living algae by aposymbiotic *H. viridis. Biol. Bull.,* **145**: 565–579.

Patton, W. K., 1967. Commensal Crustacea. *Proc. Symp. Crustacea, Ernakulam, Part* **III**: 1228–1244.

Pearce, J. B., 1962. Adaptation in symbiotic crabs of the family Pinnotheridae. *Biologist,* **45**: 11–15.

Pearce, J. B., 1966a. The biology of the mussel crab, *Fabia subquadrata,* from the waters of the San Juan archipelago, Washington. *Pac. Sci.,* **20**: 3–35.

Pearce, J. B., 1966b. On *Pinnixa faba* and *Pinnixa littoralis* (Decapoda: Pinnotheridae) symbiotic with the clam, *Tresus capax* (Pelecypoda: Mactridae). Pages 564–589 in H. Barnes, Ed., *Some Contemporary Studies in Marine Sciences.* George Allen and Unwin Ltd., London.

Polikarpov, G. G., 1966. *Radioecology of Aquatic Organisms.* North-Holland Publ. Co., Amsterdam, 314 pp.

Poole, R. W., 1974. *An Introduction to Quantitative Ecology.* McGraw-Hill, Inc., New York, 532 pp.

Rice, T. R., and R. J. Smith, 1958. Filtering rates of the hard clam (*Venus mercenaria*) determined with radioactive plankton. *Fishery Bull. 129, Fishery Bull. Fish and Wildlife Ser.,* **58**: 73–82.

Sandoz, M., and S. H. Hopkins, 1947. Early life-history of the oyster crab, *Pinnotheres ostreum* (Say). *Biol. Bull.,* **93**: 250–258.

Silas, E. G., and K. Alagarswami, 1967. On an instance of parasitism by the pea crab (*Pinnotheres* sp.) on the backwater clam (*Meretrix casta* (Chemnitz)) from India, with a review on the work on the systematics, ecology, biology and ethology of pea crabs of the genus *Pinnotheres* Latreille. *Proc. Symp. Crustacea, Ernakulam, Part* **III**: 1163–1227.

Smith, D., L. Muscatine and D. Lewis, 1969. Carbohydrate movement from autotrophs to heterotrophs in parasitic and mutualistic symbiosis. *Biol. Rev.,* **44**: 17–90.

Stauber, L. A., 1945. *Pinnotheres ostreum* parasitic on the American oyster *Ostrea virginica. Biol. Bull.,* **88**: 269–291.

Reference: *Biol. Bull.*, **148**: 68–84. (February, 1975)

BEHAVIORAL ASPECTS OF RIGHTING IN TWO ASTEROIDS FROM THE PACIFIC COAST OF NORTH AMERICA

IRWIN POLLS[1] AND J. GONOR

Oregon State University, Marine Science Center, Newport, Oregon 97365

The righting reaction in Asteroids may be defined as the ability of sea stars, when placed on their aboral surface, to turn over onto the normal position with the oral surface down.

Reese (1966) listed four basic questions concerning the righting reaction in asteroids: (1) What is the nature of the stimulus that evokes righting? (2) Is the coordination of the righting movements under central (radial and ring) or peripheral (series of reflexes) control? (3) Is there a pair of arms which tend to initiate and lead in righting, that is, is there a physiological anterior end in righting? (4) What are the sources of variation in righting?

Righting in asteroids was first studied experimentally by Vulpian (1862), Romanes and Ewart (1881), and Romanes (1885), but they were mostly concerned with the nature of the stimulus that evoked righting. Since these early studies, numerous descriptions of the righting behavior of various species of asteroids have appeared.

Detailed descriptions of the righting movements are given by Jennings (1907), Moore (1910a, 1910b, 1939), Cole (1913a), Kjerschow-Agersborg (1918), Russell (1919), Ohshima (1940), and Rodenhouse and Guberlet (1946).

The specific movements in the righting reactions of sea stars show some variability (Jennings, 1907; Ohshima, 1940), but may, in general, be classified into three basic righting methods (Ohshima, 1940). (1) Somersaulting, all five arms bend aborally bringing the tips of each arm in contact with the substratum (dorsal reflex). Typically, two adjacent arms then twist so that their oral surfaces face each other. As these two arms move to the side of the animal (walking distally), the remaining arms rise (orally) and swing over the sea star. In effect, the animal turns a somersault. (2) Folding over, three arms lower aborally. The two outer, non-adjacent, arms that have lowered twist so that their oral surfaces face each other. As these two arms move to the side of the animal, the unattached arms, instead of elevating, fold over the rest of the body. Finally, the middle arm doubles under the animal. (3) Forming the tulip, all five arms rise orally into a "budlike" shape with the aboral surface facing outward. Several arms swing over, causing the center of gravity to be changed, thereby, forcing the animal to turn over onto the normal position.

All the variations seen in asteroid righting are apparently derived from these three basic methods (Reese, 1966).

In addition to describing righting, many of these authors gave considerable attention to the question of arm preference during righting. Jennings (1907), in an extensive report on the behavior of *Astrometis sertulifera* (=*Asterias forreri*), found that the pair of arms lying immediately adjacent to the madreporite lead in righting. Similar findings have been reported by Cole (1910, 1913a, 1913b), in *Asterias forbesi,* Kjerschow-Agersborg (1918, 1922) in *Pycnopodia helianthoides,* and Russell (1919), in *Asterias gibbosa.* Cowles (1910, 1911) found that in *Echinaster crassipina,* some individuals showed a tendency to right on a specific pair of arms, which Cowles did not identify. Hamilton (1922a), working with *Pisaster ochraceus,* believed that the arm which "pulled the strongest" became the leading arm in righting. Ohshima (1940) studied righting in *Oreaster nodosus* and concluded that there was no preference for any arms in righting. Rodenhouse and Guberlet (1946) reported that *Pteraster tesselatus* showed a definite tendency to right on two adjacent arms, but that the specific pair of arms depended on individuals, not species. They also found that the leading arms were not related to the position of the madreporite. Finally, Smith (1950), noted that in *Asterias rubens,* arm preference was related to the bilateral symmetry of the larvae, rather than to greater arm length or greater number of podia.

---

[1] Present address: Metropolitan Sanitary District of Greater Chicago, Research and Development Laboratory, 5901 West Pershing Road, Cicero, Illinois 60650.

It is clear that the question posed by Reese (1966, page 174), "Does one pair of arms tend to initiate and lead in the righting movements, that is, is there a functional or physiological anterior end involved in righting?" is unsettled. This study was in part designed to investigate this question.

Much of the published work on Echinoderm behavior, especially righting, has been qualitative and no statistical study on the righting behavior of asteroids has been published. The present paper describes and compares the behavioral aspects of righting in two common rocky intertidal Oregon sea stars *Henricia leviuscula* and *Leptasterias aequalis* by quantitative methods. In addition, the significance of righting to the functional nature of the asteroid nervous system is considered.

Materials and Methods

Species studied

The asteroids *Henricia leviuscula* and *Leptasterias aequalis* were selected because they were available in adequate number, are easily maintained in the laboratory and their righting period was relatively brief. In addition, they differ morphologically.

Henricia leviuscula (Stimpson), 1857 (Order Spinulosa, Family Echinasteridae) is found along the Pacific Coast of North America from the Aleutian Islands (55° N) to San Diego, California (32° 45′N) (Feder and Christensen, 1966). This species has five long, slender, tapering arms which are cylindrical and rather rigid and stiff. *H. leviuscula* is found on intertidal rocky substrata encrusted with sponges and bryozoans (Ricketts and Calvin, 1952; Hopkins and Crozier, 1966). The largest *H. leviuscula* collected for this study measured 10.1 cm.

Leptasterias aequalis (Stimpson), 1862 (Order Forcipulata, Family Asteriidae, Subfamily Asteriinae) ranges from British Columbia (50° N) to Santa Catalina Island, California (33° 25′N) (Feder and Christensen, 1966). *L. aequalis,* a six armed sea star, is rather small, usually less than 6.0 cm in diameter. It also lives in the rocky intertidal (Ricketts and Calvin, 1952). The arms in *L. aequalis* are somewhat flattened and because the endoskeleton is less developed are more flexible and softer than those of *Henricia.*

In 1968, in June and July, 108 specimens of *H. leviuscula* and 111 specimens of *L. aequalis* were collected from rocky intertidal beaches at Yaquina Head (44° 40′N, 124° 04′W), Whale Cove (44° 47′N, 124° 04′W), and Boiler Bay (44° 50′N, 124° 04′W). Another group of 90 *H. leviuscula* were collected in May 1969 at the same places for additional studies. All available sizes of both species were collected and used in the righting trials.

The sea stars were maintained at the Oregon State University Marine Science Center in tanks of well-aerated, running sea water. Once daily measurements of the temperature of the sea water running through the holding tanks and the experimental chamber ranged from 9.5–15° C during both the 1968 and 1969 study periods. Animals were acclimated to aquarium conditions for at least 48 hours prior to study. *L. aequalis* was fed twice a week on small specimens of *Littorina.* The unfiltered laboratory sea water was considered to contain adequate organic material to maintain *H. leviuscula,* since this genus uses filter feeding (Anderson, 1960; Rasmussen, 1965).

Methods of observation

Righting behavior was examined by repeating a standard laboratory righting trial with a large number of individuals of each species, and analyzing the observations made by statistical methods.

Each of the arms of the sea stars was identified during the righting trials using the method devised by Jennings (1907) in his study of the righting reaction of *Astrometis sertulifera.* The aboral, acentric madreporite, located between two adjacent arms (Fig. 1), was used as a landmark for designating arms. By locating the madreporite at 6:00, the arms were named clockwise beginning with arm A (Fig. 1).

Righting trials were run in an aquarium with one plate glass and three plywood sides. The bottom area of the aquarium, 61 × 33 cm, was covered with a smooth plexiglass plate.

Attempts were made throughout the study to keep the environment of the observation aquarium constant. The water was 30 cm deep and continually renewed. The glass side of the observation aquarium always faced away from the laboratory window. Light during experiments was from an overhead fluorescent source. Light intensity, determined by a photographic light meter, was found to be nearly equal in all directions. Therefore, light was considered not to be a directional stimulus.

The general procedure for conducting righting trials for both species was as follows: (1) Using the observers bare hand, an individual sea star was selected from the group in the holding tank and placed in the observation aquarium with its oral surface in contact with the plexiglass plate. (2) After a few minutes, the tip of one arm was grasped with the fingers and the sea star inverted under water. (3) The righting reaction was observed from above and from the side of the aquarium and timed. (4) The sea star was transferred to a separate holding tank and not used again that day, except in the time-size studies described separately.

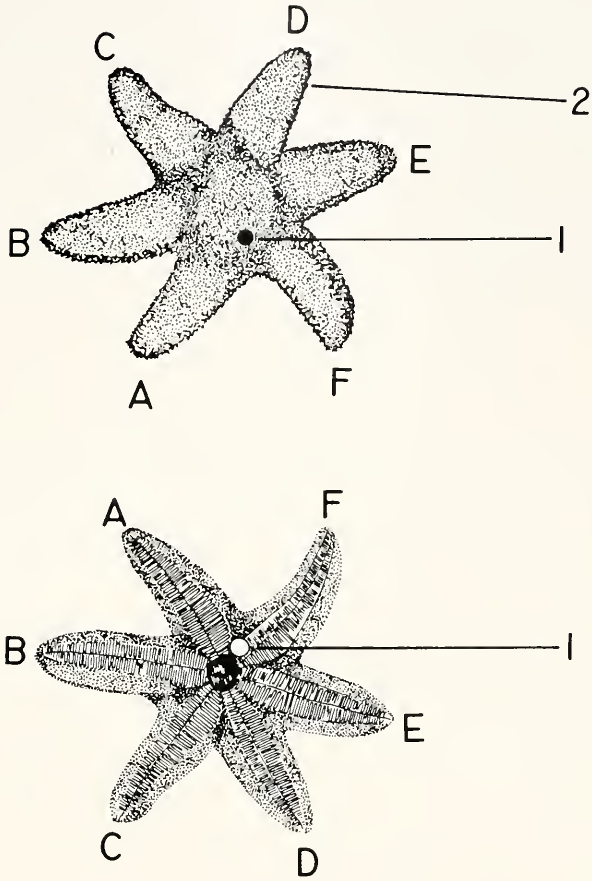


FIGURE 1. Letter designation of arms of *Leptasterias aequalis*; upper diagram, aboral surface, lower diagram, oral surface (ventral), with the position of the madreporite on the aboral surface outlined; 1. madreporite; 2., arm or ray.

Separate trials were not conducted for each of the phenomena being investigated. Instead, during each trial timed observations were made on righting method, pre-righting activity, arm movements and identity of leading arms.

Between July 15 and August 4, 1968, when most of this work was done, 130 trials were conducted using the group of 108 specimens of *H. leviuscula* and 260 trials conducted using the 111 specimens of *L. aequalis* available. During the 1968 trials, the temperature in the observation aquarium was measured intermittently and found to range from 9.5–15° C.

The relationship between righting time and individual size was investigated in both species by additional sets of trials. Approximately ten individuals of each of three size classes were selected for each species. Size classes were based on arm length, or radius (R), measured as the distance from the tip of the arm to the center of the mouth.

Procedures for all time-size trials were as described above, except that each individual was used for three trials, designated A, B and C, with six and 12 hour rest intervals between successive trials. A total of 84 time-size trials were run for each species.

In the case of *L. aequalis* 28 individuals were selected from the 1968 group used in previous trials. Small specimens of *L. aequalis* measured 0.8–1.9 cm, medium, 2.1–2.9 cm, and large, 3.0–4.1 cm. In 1969, 28 specimens of *H. leviuscula* were selected from the 90 individuals available. Small specimens of *H. leviuscula* measured 0.4–2.8 cm, medium, 3.1–4.8 cm, and large, 5.0–10.1 cm.

The data were tested statistically using either the chi-square "goodness-of-fit" test or the Student's "t" test at the 0.05 level of significance.

RESULTS

Righting methods

H. leviuscula used only the somersaulting method to right itself. *L. aequalis* used both the folding over and somersaulting methods when righting. The somersaulting method was used significantly more than folding over ($X^2 = 132$; d.f. $= 1$; $p = 0.005$). Occasionally *L. aequalis* used a combination of both folding over and somersaulting methods in righting.

Pre-righting period

According to previous studies, when a sea star is first placed on its back, it usually rests on its disc because the aboral surface is convex. It then remains motionless for a variable amount of time before a reaction appears. This is called the latent or quiescent period.

No entirely motionless quiescent period was observed in *H. leviuscula* and *L. aequalis*, neither of which are markedly convex. In both species, some arm and tube foot movements could be detected immediately after inversion. These movements were slight and very difficult to observe, but small, jerking movements by some of the arms were always detected. These slight movements lasted for 0.5 to 3 seconds before major arm movements occurred.

Arm movements

Following the pre-righting period, arm activity increases and leads eventually to resumption of the normal position with the oral surface and tube feet in contact with the substratum. The observations on *H. leviuscula* and *L. aequalis* showed that the chief arm movements used in righting were: (1) Lowering (aboral flexure)—when a sea star was first turned over and placed on its back, the aboral surfaces of the arms were not in contact with the substratum, but slightly elevated above it. The lowering activity permits an arm to come in contact with the substratum. (2) Raising (oral flexure)—raising occurred when an arm rose from the substratum so that it was above the animal with its aboral surface facing away from the mouth and the tube feet facing inward. The tip of the arm was held above the animal. (3) Rolling (twisting)—an arm can be described as rolling when it twists on its axis to either side, bringing its tube feet in contact

Table I

Statistical analysis comparing the leading arms used in righting in Henricia leviuscula *utilizing the chi-square goodness-of-fit test*

Leading arms compared	N*	Degrees of freedom	Computed X^2 values	Probability of a larger X^2
C and A	106	1	4.56	0.050**
C and B	110	1	2.94	0.100
C and D	127	1	0.007	0.950
C and E	109	1	3.31	0.100
D and A	105	1	4.20	0.050**
D and B	109	1	2.65	0.110
D and E	108	1	3.00	0.100

* Total number of times the two arms are used as leading arms, based on 130 trials.

** Significantly different at the 5% level.

with the substratum. All possible combinations of the major righting movements, lowering, raising, and rolling of the arms, resulted in the sea star turning over.

Detailed description of righting reactions

In *H. leviuscula*, during the pre-righting period the ambulacral grooves opened wide and the tube feet were extended. Next, the tube feet on one arm all extended in one direction. As the reaction proceeded, the other four arms were lowered and eventually brought their aboral surfaces in contact with the substratum, and their tube feet pointed in the same direction as those of the arm first lowered. While the arms were being lowered, they were usually also rolling. Once the substratum had been gripped, four or five arms (one sometimes temporarily remaining inactive) began to roll to one side or the other. Eventually, two arms rolled so that their ventral surfaces faced each other. They then began "walking" to the side of the animal bringing more and more tube feet into action. These two arms are called the leading arms in this discussion. As these two arms moved to the side of the animal, one or two of the remaining arms released themselves and swung over the sea star until their ventral surfaces reached the bottom. The other arm(s) was then pulled over, so that the animal in effect turned a somersault.

L. aequalis used either the folding over or somersaulting method when righting. Occasionally, a combination of both righting methods was used. Following the pre-righting period, five or six arms were lowered, bringing their aboral surfaces in contact with the substratum. As these arms lowered they were usually rolling distally. Once the substratum had been gripped, three to six arms began rolling to either side. The lowering and rolling phases were followed by one of the three types of reactions described below.

First, two of the arms that were rolling on the substratum eventually twisted so that their ventral surfaces faced each other. Except for the fact that one additional arm swung over, the rest of the righting reaction was very similar to the righting method described for *H. leviuscula.*

Secondly, three adjacent arms that were rolling on the substratum twisted so that the ventral surfaces of the outer arms eventually faced each other. These became the leading arms. The arm between the two leading arms remained pas-

TABLE II

Statistical analysis comparing the leading arms used in righting in Leptasterias aequalis *utilizing the chi-square goodness-of-fit test*

Leading arms compared	N†	Degrees of freedom	Computed X^2 values	Probability of a larger X^2
A and B	194	1	0.08	0.900
A and C	176	1	2.75	0.100
A and D	165	1	6.66	0.010**
A and E	189	1	0.42	0.750
A and F	192	1	0.19	0.750
B and C	172	1	1.88	0.250
B and D	161	1	5.23	0.025*
B and E	185	1	0.14	0.750
B and F	188	1	0.01	0.900
C and D	143	1	0.84	0.500
C and E	167	1	1.01	0.500
C and F	170	1	1.51	0.250
D and E	156	1	3.69	0.100
D and F	159	1	4.58	0.050*
E and F	183	1	0.05	0.900

† Total number of times the two arms are used as leading arms, based on 260 trials.
* Significantly different at the 5% level.
** Significantly different at the 1% level.

sive and later doubled under the disc or walked backwards under the sea star. As the two leading arms moved to the side of the animal, one, two, or three of the remaining arms released and swung over the sea star. Finally, the other arms were pulled over until their ventral surfaces reached the substratum.

Thirdly, four adjacent arms that were rolling on the substratum eventually twisted so that the ventral surfaces of the two outer arms faced each other. The two middle arms either remained passive and later doubled under the disc or walked backwards under the animal. The two remaining unattached arms were finally pulled over by the action of the four attached arms.

In *L. aequalis*, the coordinated tube foot movements of all arms appeared later in the righting reaction than in *H. leviuscula*. In both species this unified response was the first visible sign of coordination of all arms to produce the righting movement.

Leading arms

In order to determine whether or not *H. leviuscula* and *L. aequalis* showed a preference for using a particular arm or combination of arms to turn over in the righting reaction, the data were subjected to statistical analysis by the chi-square goodness-of-fit test. The results are summarized in Tables I to IV.

For the statistical analysis each arm was first considered separately, even when functioning as a member of a pair of leading arms.

TABLE III

Statistical analysis comparing the leading pairs of arms used in righting in Henricia leviuscula *utilizing the chi-square goodness-of-fit test*

Leading pairs of arms compared	N*	Degrees of freedom	Computed X^2 values	Probability of a larger X^2
C/D and A/B	60	1	5.40	0.025**
C/D and B/C	64	1	3.08	0.100
C/D and D/E	63	1	3.57	0.100
C/D and E/A	60	1	5.40	0.025**

* Total number of times the pairs of arms were used as leading pairs of arms, based on 130 trials.
** Significantly different at the 5% level.

From the data presented in Table I it can be noted that *H. leviuscula* used arms C and D more often as leading arms than arms A, B, and E. When arms C and D were compared they were found to be used as leading arms equally. Only when arms C and D were compared with arm A were they found to be used significantly more often as leading arms.

Analysis of the data on leading arms in *L. aequalis,* indicated that in this species also, certain arms were used more as leading arms than other arms. The results are shown in Table II. Arms A, B, E and F were used more as leading arms in the righting reaction than arms C and D. Also, the data showed that arms A, B, E and F were all used equally as leading arms, as were arms C, and D. Only when arms A, B, and F were compared to arm D were they found to be used significantly more often as leading arms.

It was previously shown that, when righting, both species preferred the variation of the somersaulting method using two adjacent arms. The next step in the analysis was to determine whether or not either species showed preference for any pair of adjacent arms. In 15% of the 260 trials, *L. aequalis* used three or four arms as a leading group when using the folding over method. These data were not used in the analysis of pair preference.

In *H. leviuscula* the combination of arms C/D was used more as a leading pair of arms than the pairs A/B, B/C, D/E, and E/A (Table III). Only when the pair of arms C/D was compared with the combination of arms A/B, and E/A, was it found to be used significantly more often as a leading pair of arms.

Analysis of the data on leading pairs of arms in *L. aequalis* indicated that the pair or combination of adjacent arms A/B, B/C, E/F, and F/A were used more often as leading arms in the righting reaction than arms C/D and D/E (Table IV). The pair of arms C/D and D/E were used equally as often as leading arms in the righting reaction, as were the pairs A/B, B/C, E/F and F/A. Only when the pairs of adjacent arms A/B, B/C, and E/F were compared to C/D were they found to be used significantly more as a leading pair of arms.

Time

The time required for righting in *H. leviuscula* was very variable, ranging from 94 to 570 seconds. The average time taken was 230 seconds. In *L. aequalis,* variability in righting time ranged from 67 to 664 seconds for the somersaulting

TABLE IV

Statistical analysis comparing the leading pairs of arms in righting in Leptasterias aequalis *utilizing the chi-square goodness-of-fit test*

Leading pairs of arms compared	N*	Degrees of freedom	Computed X^2 values	Probability of a larger X^2
A/B and B/C	88	1	0.00	0.000
A/B and C/D	66	1	7.30	0.010**
A/B and D/E	74	1	2.64	0.250
A/B and E/F	87	1	0.00	0.900
A/B and F/A	81	1	0.60	0.500
B/C and C/D	66	1	7.30	0.010**
B/C and D/E	74	1	2.64	0.250
B/C and E/F	87	1	0.01	0.900
B/C and F/A	81	1	0.60	0.500
C/D and D/E	52	1	1.23	0.500
C/D and E/F	65	1	6.80	0.010**
C/D and F/A	59	1	3.81	0.100
D/E and E/F	52	1	2.26	0.250
D/E and F/A	67	1	0.70	0.500
E/F and F/A	80	1	0.45	0.500

* Total number of times the pair of arms were used as leading pairs of arms, based on 220 trials.
** Significantly different at the 1% level.

method and from 100 to 623 seconds for the folding over method. The average time taken was 168 seconds for the somersaulting method and 195 seconds for folding over. Analysis of the data showed that there was no significant difference in righting time between the somersaulting and folding over method in *L. aequalis* ($X^2 = 2.01$; d.f. = 1; $P = 0.20$).

Time-size relationship

In order to determine whether there was a significant relationship between body size (the R measurement) and the righting time in *H. leviuscula* and *L. aequalis,* the variance, standard deviation, and standard errors of the righting time was determined for each of the three size categories for both species (Tables V and VI). Righting time ranged from as much as 400 seconds in large individuals to 200 seconds in smaller individuals.

For each species the mean righting times of the three size classes were compared using the t-test. In *H. leviuscula* (Table VII) a significant difference was found between the righting times of small and medium sized individuals (t = 2.79; d.f. = 26; $P = 0.005$), and medium and large sized individuals (t = 6.64; d.f. = 26; $P = 0.005$). In *L. aequalis,* when small and medium sized individuals were compared, it was found that there was a significant difference between their righting times (t = 4.39; d.f. = 26; $P = 0.005$). The same was true of medium and large sized individuals (t = 1.71; d.f. = 26; $P = 0.10$). These facts suggest that

TABLE V

Summary of statistical analysis of the righting times in Henricia leviuscula *of different sizes*

Trial	Sum (Seconds)	$\bar{y}$	Range	S^2	s	S.E.	N*
Small (R = 0.4–2.8 cm)							
A	1019	113	81–168	806	28	9.46	9
B	1378	153	86–230	3,145	56	18.70	9
C	1394	155	127–196	796	28	9.40	9
Total	3791	140	81–230	1,849	43	8.26	27
Medium (R = 3.1–4.8 cm)							
A	1766	177	100–412	7,951	89	27.90	10
B	1823	182	84–323	7,809	88	27.63	10
C	2014	201	128–307	3,985	63	19.72	10
Total	5603	187	84–412	6,244	79	14.36	30
Large (R = 5.0–10.1 cm)							
A	2776	308	165–426	9,877	99	33.10	9
B	3283	365	233–524	12,895	114	38.00	9
C	3421	380	274–554	9,509	98	32.66	9
Total	9480	351	165–554	10,918	105	20.99	27

* Total number of times animals were turned over

there was a significant relationship between the body size and the righting time in both species of sea stars, with smaller individuals righting faster than larger.

It was also observed that *H. leviuscula* took longer times to right in successive trials, even with six and 12 hour rest intervals between trials (Table V). This was not true of *L. aequalis* (Table VI).

Mechanical stimulation

Sea stars were turned over by picking them up by the tip of one arm. The possible effect on the frequency of the use of such arms as lead arms was studied by analyzing the data with the chi-square goodness-of-fit test.

Regardless of which arm was used to turn *H. leviuscula* over, the arm so stimulated was used with the same frequency as a leading arm as each of the other arms. Therefore, in *H. leviuscula* mechanical stimulation did not affect the use of an arm as a leading arm in any way.

On the other hand, analysis of the effects of mechanical stimulation on *L. aequalis* indicated that regardless of which arm was used to turn the sea star over, the arm so stimulated was used less as a leading arm than each of the other arms.

Further statistical analysis on the effect of mechanical stimulation showed that in most instances the arm used to turn the sea star over was used less as a leading arm than it would have been if that arm had not been used to turn the sea star over and less than each of the other arms.

TABLE VI

Summary of statistical analysis of the righting times in Leptasterias aequalis *of various sizes*

Trial	Sum (Seconds)	$\bar{y}$	Range	S^2	s	S.E.	N*
Small (R = 0.8–1.9 cm)							
A	1160	145	86–236	3,441	59	20.85	8
B	1604	160	95–379	7,211	85	26.87	10
C	1223	136	83–269	3,263	57	19.03	9
Total	3987	148	83–379	4,536	67	12.45	27
Medium (R = 2.1–2.9 cm)							
A	3028	303	123–576	14,767	122	38.40	10
B	2127	213	157–407	5,030	71	22.44	10
C	2332	233	156–484	9,837	99	31.39	10
Total	7487	250	123–576	10,735	104	18.92	30
Large (R = 3.0–4.1 cm)							
A	3127	347	199–737	29,374	171	57.13	9
B	2285	254	151–585	24,161	156	51.83	9
C	2970	330	183–694	30,537	175	58.23	9
Total	8479	310	151–737	35,494	188	36.26	27

* Total number of times animals were turned over

DISCUSSION

Variation in the righting method has been attributed to many extrinsic and intrinsic factors (Reese, 1966). In this work, the methods of study and maintenance of laboratory animals were designed so that as many of these factors as possible were kept constant, or variation in them kept minimal.

Somersaulting characterized the righting method employed by both *H. leviuscula* and *L. aequalis*. In contrast, the folding over method, and a combination of folding over followed by somersaulting were used only by *L. aequalis*. Table VIII summarizes the various righting methods known to be used by asteroids, and demonstrates that the method used by *H. leviuscula* and *L. aequalis* is the predominate method known.

Since representatives of all major extant orders are included, the somersaulting method described in detail here appears to be characteristic of the entire Class Asteroidea.

Evidence for the existence of a quiescent period in righting in asteroids is given by Moore (1939), Ohshima (1940), Hyman (1955), and Reese (1966). The present study indicates that *H. leviuscula* and *L. aequalis* are active during the pre-righting period, suggesting that there is no true quiescent period in these species. Evidence was found by the author that *Astropecten brasiliensis armatus* and *Patiria miniata* (Polls, unpublished data) also have no quiescent period.

Table VII

Statistical analysis comparing the righting times of various size categories of Henricia leviuscula *and* Leptasterias aequalis *utilizing the t-test*

Species	Size categories compared	N†	Degrees of freedom	Computed t value	Probability of a larger t
Henricia leviuscula	Small and Medium	57	26	2.79	0.005**
	Medium and Large	57	26	6.64	0.005**
Leptasterias aequalis	Small and Medium	57	26	4.39	0.005**
	Medium and Large	57	26	1.71	0.050*

† Total number of times animals were turned over.
* Significantly different at the 5% level.
** Significantly different at the 1% level.

Due to the shortness of the pre-righting period, no attempt was made to discover which arms were actually moving first, nor how this might affect the role played by these individual arms during the rest of the righting reaction. Additional observations are needed to answer questions on the specific order and nature of the pre-righting motions, and their significance.

During the pre-righting period, the sea star may be sensing its inverted position and relaying this sensory information by way of the nervous system to other parts of the body. Apparently, once the inverted orientation has been detected and nervous integration accomplished, the righting reaction can be initiated.

Following the pre-righting period, the tube feet of all the arms are extended outward in many directions. Once the initial, oriented, tube feet movements appear, all activities of the sea star are committed towards turning in a certain unified way and direction. The establishment of righting response implies a coordinated activity of all the arms and the tube feet.

Coordinated arm movements were clearly present in *H. leviuscula* at the beginning of the righting reaction but, in *L. aequalis*, they appeared later. Early coordinated movements have also been observed in other asteroids by Jennings (1907), Cole (1913b), Kjerschow-Agersborg (1918), Russell (1919), Hamilton (1922b), and Moore (1939).

Jennings (1907) lists a number of factors which may determine the direction of the righting movements, the most important of which is the tendency to right with a fixed pair of arms. Once the tube feet of these arms are all extended in the same direction, these arms become the leading arms. In other words, once the righting response appears, one can tell early in the righting reaction which will be the leading pair of arms.

The results of this study show that following the righting response, the attached tube feet of the non-leading arms let go (releasing response) and extend in the direction of the leading arms.

Hamilton (1922b), Moore (1945), Kerkut (1954), and Smith (1965) also found that following the righting response the tube feet of the subordinate arms detach. Suppression of activity by the tube feet of non-leading arms thus plays a critical part in the first movements of the righting reaction. No causal explana-

TABLE VIII

Righting methods used by asteroids

Order	Species	Most Common Righting Methods	Investigator
Phanerozonia	*Astropecten auranciacus*	Somersaulting and tulip	Romanes (1885)
	Astropecten brasiliensis armatus	Tulip	Polls (unpublished)
	Oreaster nodosus	Folding over	Ohshima (1940)
Spinulosa	*Asterina gibbosa*	Somersaulting and folding over	Russell (1919)
	Henricia leviuscula	Somersaulting	Polls (1969)
	Patiria miniata	Somersaulting	Polls (unpublished)
	Pteraster tesselatus	Somersaulting	Rodenhouse and Guberlet (1946)
Forcipulata	*Asterias forbesi*	Folding over	Cole (1913a, 1913b)
	Asterias rubens	Somersaulting	Romanes and Ewart (1881) Romanes (1885) Smith (1950)
	Astrometis sertulifera	Somersaulting and folding over	Jennings (1907)
	Leptasterias aequalis	Somersaulting	Polls (1969)
	Pisaster ochraceous	Somersaulting	Moore (1910a, 1910b) Hamilton (1921, 1922a, 1922b)
	Pycnopodia helianthoides	Combination of somersaulting and folding over	Kjerschow-Agersborg (1918)

tion for this suppression, which must require central nervous system integration, can be given at this time.

Many investigators have attempted to discover whether there is a physiological anterior end involved in righting. The literature dealing with this question falls into two major categories. Ohshima (1940) found no dominant or leading arms in righting, and consequently no anterior end. In contrast, Jennings (1907), Cole (1910, 1913a, 1913b), Cowles (1910, 1911), Kjerschow-Agersborg (1918, 1922), Russell (1919), Hamilton (1922a), Rodenhouse and Guberlet (1946), Smith (1950), and Polls (unpublished data) reported that there is a physiological anterior end marked by a dominant pair of arms.

Since the results of this study show that there was a leading pair of arms in both *H. leviuscula* and *L. aequalis* they support the idea that sea stars have a physiological anterior end. It is not known why sea stars use one combination of arms more frequently than others as a leading pair, nor why different species use different pairs.

Reese (1966, page 179), after reviewing the literature on righting in Echinoderms, concludes that "there is a tendency for one pair of arms—usually at least one arm of the pair is adjacent to the madreporite—to lead the righting movement." However, he further says that this preference for one pair of arms is an individual rather than a species characteristic.

In this study it was not possible to follow the behavior of individuals since various marking techniques such as vital dyes and tagging were unsuccessful. Although some sea stars may exhibit individual preference for a leading arm, the results of this study show that in *H. leviuscula* and *L. aequalis* the preference for certain arms to lead in the righting reaction is a species rather than an individual characteristic.

While *H. leviuscula* and *L. aequalis* showed a preference for certain arms to lead in the righting reaction, these are not adjacent to the madreporite. The position of the madreporite may have indirect significance in arm preference during righting in asteroids. Unfortunately, there is no information in the literature on how the madreporite is related to the central nervous system, nor is there any obvious asymmetry of the nervous system in the madreporite region. The madreporite remains a curious bench-mark.

It was observed that in *H. leviuscula* and *L. aequalis*, the preference for a specific arm is not due to the length of the arm, or greater number of tube feet as suggested by Hamilton (1922a), since the leading arms were the same length as the non-leading arms. This also was found by Cole (1913a) in *Asterias forbesi*, Crozier (1920) in *Coscinasterias tenuispina*, and Smith (1950) in his study of *Asterias rubens*.

The time required to right from mechanical inversion to the natural posture varied considerably in both *H. leviuscula* and *L. aequalis*. Variability in the righting time has also been reported by Cole (1913b), Ohshima (1940), and Rodenhouse and Guberlet (1946).

Kleitman (1941) found that there was a relationship between righting time and temperature, with righting time increasing as the temperature rose. The temperature was not controlled during the trials in this study. The possible effect of the approximately 5° C temperature variation between experiments was not analyzed. Analysis of the data shows a strong relationship between size and time. Any possible effects of temperature were probably less than the effect of size on time of righting.

Asterias, Asterina, Leptasteria, and *Pycnopodia* have soft, flexible bodies, slender arms (except *Asterina*), and strong tube feet. The species studied in these genera can right quite rapidly. Cole (1913b) reported that the average time for *Asterias forbesi* was 160 seconds. Russell (1919) found it to be 30 to 45 seconds for *Asterina gibbosa*. For *L. aequalis*, the average time was 181 seconds. Kjerschow-Agersborg (1918) reported that the average time for *Pycnopodia helianthoides* to be 57.1 seconds.

In contrast, *Henricia* and *Oreaster* have stiff, non-flexible bodies, the tube feet are weak, and they take much longer to right than other species. The average time taken to right for *H. leviuscula* was 230 seconds. Ohshima (1940) discovered it to be 360 to 420 seconds for *Oreaster nodosus*. Differences in righting time between species appear to be primarily an expression of differences in morphology rather than of environmental variables or behavioral modes.

The great variation observed in the righting time in both species studied raised the question of the possible relationship between the size of the sea stars and their righting times. The statistical analysis showed that there was a significant relationship between body size and righting time ($P = 0.005$) for both species. This suggests that with an increase in body size there is an increase in the length of time taken to right within a species of asteroid.

Therefore, since a relationship has been shown between righting time and body size, it is probable the average righting times given for other species of asteroids may have been influenced by the size of the sea stars used. Unfortunately, no size categories were given for the sea stars used in previous time-study investigations.

It was also observed that after six or even 12 hour rest intervals before being turned over again, *H. leviuscula* took a longer time to right. The same phenomenon was not observed in *L. aequalis*. Kjerschow-Agersborg (1918) and Ohshima (1940) found after repeated trials, the righting time increased, which they interpreted as fatigue. Jennings (1907) noted that after repeated turning over of the same individual, there was no improvement in the time taken to right.

It is possible that the increase in righting time observed in *H. leviuscula, Pycnopodia helianthoides,* and *Oreaster nodosus,* may be due to a process similar to habituation.

The statistical analysis demonstrated that in *L. aequalis,* the arm used to turn the sea star over was used less frequently as a leading arm. No such effects of mechanical stimulation on a leading arm were observed in *H. leviuscula.*

This evidence supports the suggestions of Jennings (1907) and Smith (1950) that an arm used most frequently as a leading arm is conditioned by imposed external stimulation. Smith (1950) also states that intensive stimulation of leading arms causes that arm to be used less as a leading arm. Similar observations have been noted by Polls (unpublished data) in *Patiria miniata.*

These findings suggest that if previous investigators turned over the sea stars by the same arm on each trial, then perhaps the leading arms they found were not the true leading arms. In many reports on righting in asteroids, no mention is made as to which arm(s) was used to turn the sea stars over.

H. leviuscula and *L. aequalis* live on intertidal rocky shore areas which are exposed to heavy wave action. Therefore, a righting reaction in this habitat is of obvious survival advantage.

The statistical analysis demonstrated that there was no difference in the righting time between the somersaulting and folding over method in *L. aequalis.* Therefore, neither method would appear to be more advantageous for survival in nature. Field studies on the frequency and circumstances of use of the two methods may reveal their significance.

The authors wish to thank Dr. R. B. Willey, Dr. R. G. Peterson, and Dr. John S. Pearse for help and suggestions, and Dr. E. S. Reese for his critical reading of the manuscript.

SUMMARY

1. The behavioral aspects of righting were studied in the asteroids *Henricia leviuscula* and *Leptasterias aequalis* by detailed observations on the righting method, including leading pair of arms and righting time.

2. Somersaulting characterized the righting method employed by both species. In contrast, the folding over method, and a combination of folding over followed

by somersaulting were used only by *L. aequalis.* Somersaulting appears to be characteristic of the entire class Asteroidea.

3. Both species are active during the pre-righting period, suggesting that there is no entirely motionless quiescent period.

4. The righting response was clearly present in *H. leviuscula* at the beginning of the righting reaction. However, in *L. aequalis,* it appeared later.

5. *H. leviuscula* exhibited a tendency to utilize arms C/D more as the leading pair of arms than other arm pairs. In *L. aequalis,* arm pairs A/B, B/C, E/F, and F/A were used more as leading arms than arm pairs C/D and D/E. In both species the preference for certain arms to lead in righting reaction is a species rather than an individual characteristic. Leading arms are not adjacent to the madreporite.

6. The time required for righting in both species was very variable. Differences in righting time between species appears to be an expression of differences in morphology rather than of environmental variables or behavioral modes.

7. There was a significant relationship between the body size and the righting time in both species of sea stars, with smaller individuals righting faster than larger.

8. Mechanical stimulation affected the arms used in righting in *L. aequalis.* The arm used to turn the sea star over was used less frequently as a leading arm. It is possible that the results of some previous studies are in error because of this response. However, no such effects were observed in *H. leviuscula.*

9. Since there was no difference in the righting time between the somersaulting and folding over method in *L. aequalis,* neither method would appear to be more advantageous for survival in nature.

LITERATURE CITED

Anderson, J. M., 1960. Histological studies on the digestive system of a starfish, *Henricia,* with notes on Tiedemann's pouches in starfishes. *Biol. Bull*, **119**: 371–398.

Cole, L. J., 1910. Direction of locomotion of the starfish (*Asterias forbesi*). *Science,* **31**: 474.

Cole, L. J., 1913a. Direction of locomotion of the starfish (*Asterias forbesi*). *J. Exp. Zool.,* **14**: 1–32.

Cole, L. J., 1913b. Experiments on coordination and righting in the starfish. *Biol. Bull.,* **24**: 362–369.

Cowles, R. P., 1910. Preliminary report on the behavior of echinoderms. Pages 128–129 in *Eighth Yearbook of the Carnegie Institution of Washington, 1909.* Washington, D.C.

Cowles, R. P., 1911. Reaction to light and other points in the behavior of starfish. *Papers Tortugas Laboratory Carnegie Institution, Washington,* **3** (# 132): 95–110.

Crozier, W. J., 1920. Notes on some problems of adaptation. 2. On the temporal relations of asexual propagation and gametic reproduction in *Coscinasterias tenuispina:* with a note on the direction of progression and on the significance of the madrepores. *Biol. Bull.,* **39**: 116–129.

Feder, H. M., and A. M. Christensen, 1966. Aspects of asteroid biology. Pages 87–127 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience Publishers, New York.

Hamilton, W. F., 1921. Coordination of the starfish. I. Behavior of individual tube feet. *J. Comp. Psychol.,* **1**: 473–488.

Hamilton, W. F., 1922a. Coordination of the starfish. II. Locomotion. *J. Comp. Psychol.,* **2**: 61–75.

Hamilton, W. F., 1922b. Coordination in the starfish. III. The righting reaction as a phase of locomotion. *J. Comp. Psychol.,* **2**: 81–94.

Hopkins, T. S., and G. F. Crozier, 1966. Observations on the asteroid echinoderm fauna occurring in the shallow water of Southern California. *Bull. Southern Calif. Acad. Sci.*, **65**: 129–145.

Hyman, L. H., 1955. *The Invertebrates: Echinodermata.* McGraw-Hill, New York, 763 pages.

Jennings, H. S., 1907. Behavior of the starfish *Asterias forreri de Loriol. University of California Pub. Zool.*, **4**: 53–185.

Kerkut, G. A., 1954. The mechanisms of coordination of the starfish tube feet. *Behavior*, **6**: 206–232.

Kjerskog-Agersborg, H. P., 1918. Bilaterial tendencies and habits in the twenty-rayed starfish *Pycnopodia helianthoides* (Stimpson). *Biol. Bull.*, **35**: 232–254.

Kjerchow-Agersborg, H. P., 1922. The relation of the madreporite to the physiological anterior end in the twenty-rayed starfish, *Pycnopodia helianthoides* (Stimpson). *Biol. Bull.*, **42**: 202–216.

Kleitman, N., 1941. The effect of temperature on the righting of echinoderms. *Biol. Bull.*, **80**: 292–298.

Moore, A. R., 1910a. On the nervous mechanism of the righting movements of the starfish. *Amer. J. Physiol.*, **27**: 207–211.

Moore, A. R., 1910b. On the righting movements of the starfish. *Biol. Bull.*, **19**: 235–239.

Moore, A. R., 1939. Injury, recovery, and function in an aganglionic central nervous system. *J. Comp. Psychol.*, **28**: 313–334.

Moore, A. R., 1945. *The Individual in Simpler Forms.* University of Oregon Monographs, Studies in Psychology, #2.

Ohshima, H., 1940. The righting movements of the sea-star *Oreaster nodosus* (Linné). *Jap. J. Zool.*, **8**: 575–589.

Polls, I., 1969. Behavioral aspects of righting in the asteroid echinoderms *Henricia leviuscula* (Stimpson) and *Leptatasterias aequalis* (Stimpson). *Masters thesis, Oregon State University, Corvallis*, 65 pp.

Rasmussen, B. N., 1965. On taxonomy and biology of the North Atlantic species of the asteroid genus *Henricia* Gray. *Meddr, Danm. Fisk.-og Havunders.*, **4**: 157–213.

Reese, E. S., 1966. The complex behavior of echinoderms. Pages 157–218 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience Publishers, New York.

Ricketts, E. F., and J. Calvin, 1952. *Between Pacific Tides.* Stanford University Press, Stanford, 502 pp.

Rodenhouse, I. Z., and J. E. Guberlet, 1946. The morphology and behavior of the cushion star *Pteraster tesselatus* Ives. *Univ. Wash. Publ. Biol.*, **12**: 21–47.

Romanes, G. J., 1885. *Jelly-fish, Star-fish, and Sea-Urchins.* Appelton, New York.

Romanes, G. J., and J. C. Ewart, 1881. Observations on the locomotor system of the *Echinodermata. Phil. Trans. Roy. Soc. London Series B*, **172**: 829–885.

Russell, E. S., 1919. Note on the righting reaction in *Asterina gibbosa Penn. Proc. Zool. Soc. London*, **1919**, 423–432.

Smith, J. E., 1950. Some observations on the nervous mechanisms underlying the behavior of starfishes. *Symp. Soc. Exp. Biol.*, **4**: 196–220.

Smith, J. E., 1965. Echinodermata, Pages 1520–1558 in T. H. Bullock and G. A. Horridge, Eds., *Structure and Function in the Nervous Systems of Invertebrates.* Freeman, San Francisco.

Vulpian, A., 1862. Recherches expérimentales sur la tendance à l'attitude normale chez les astéries. *Comptes Rendus Séances Memories Société Biologie*, **3**: 189–196.

Reference: *Biol. Bull.*, **148**: 85–105. (February, 1975)

ACTIVE BRANCHIAL AND RAM GILL VENTILATION IN FISHES[1]

JOHN L. ROBERTS

Department of Zoology, The University of Massachusetts, Amherst, Massachusetts 01002; and Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Fish biologists have long known that adult mackerel and tunas (Scombridae) can be kept alive only in tanks of a shape and size that permit continuous swimming at speeds just over 1 km·hr^{-1}. Hall (1930) dramatically demonstrated the physiological basis for this observation in 1930. He found Atlantic mackerel, *Scomber scombrus*, slowed in swimming by towing erlenmeyer flasks could not maintain normal blood-oxygen saturation values as could unimpeded fish. These results and the absence of visible ventilation movements in adult swimming mackerel led him to conclude that open-mouth swimming (ram gill ventilation) is routine for this fish, and that the ability to effectively ventilate gills by mouth and opercular pumping had been lost.

Now it is known that other pelagic and mid-water fishes can readily suspend active breathing when on the move at velocities about 1.5 km·hr^{-1} or higher and also resort to ram ventilation of the gills (Muir and Buckley, 1967, remora; Smith, Brett and Davis, 1967, sockeye salmon; Roberts, 1970, 1974, four marine species). Yet these other fishes, unlike scombrids, retain the ability to ventilate at rest in the water column or when maneuvering at slow speed. In other words, the mackerel-like fishes seem to have "outgrown" the capacity of their active ventilatory systems (buccal and opercular pumps) to oxygenate the blood as adults so that continuous swimming becomes a survival necessity.

Many factors tied to the life styles evolved by different lines of fishes played significant roles in the development of the ability to transfer from active to ram gill ventilation when swimming speed picks up from rest (Hughes, 1960a; Hughes and Shelton, 1962). Most crucial must have been the relatively higher metabolic cost of oxygen uptake from water compared to air; a cost largely due to the low solubility of oxygen and the work of pumping water, a respiratory medium of high viscosity and density (Hughes and Roberts, 1970; Randall, 1970b). Consequently, it seems decidedly of advantage for a good swimmer to switch from active to ram ventilation as it grows to a size at which it can routinely cruise fast enough to overcome gill flow resistance, but still ensure complete blood oxygenation. This analysis substantiates this view with detailed and updated experiments. A brief resumé of these was reported earlier (Roberts, 1970).

MATERIALS AND METHODS

Experiments were completed with the five species of fishes listed in Table I. They were obtained by trapping in Buzzards Bay by the aquarium staff, National

---

[1] This investigation was supported by research grant, GB 8022, from the National Science Foundation, and by a NRC-NOAA Senior Research Associateship from the National Research Council.

TABLE I

Species used to determine swimming velocities for conversion from active to passive gill ventilation

Species	N	Standard length, cm (range)	Weight, g (range)	Experimental temperature, °C
Atlantic mackerel *Scomber scombrus*	5	19.1 (15.8–20.0)	71.9 (43.0–83.3)	18.5–22.0
Blue runner *Caranx crysos*	7	19.2 (17.6–20.5)	153.3 (114–178)	18.5–19.5
Bluefish *Pomatomus saltatrix*	11	19.3 (17.5–22.0)	105.2 (62.9–141)	16.0–19.5
Northern scup *Stenotomus crysops*	5	17.2 (14.5–18.5)	102.4 (72.2–115)	19.5–20.0
Striped mullet *Mugil cephalus*	4	20.5 (19.3–21.4)	101.7 (90.6–110)	20.0–20.5

Marine Fisheries Service, Woods Hole, Massachusetts, or by bait and lure casting off the stone jetty in Woods Hole Harbor. The blue-runners, scup, and mullet were young adults of smaller than average size. The mackerel and bluefish juveniles were of a size commonly called "tinker" mackerel and "snapper blues." Except for the mackerel that were used soon after capture at the capture temperature, the fish were kept in the large holding tanks of the aquarium in running sea water. To some extent, the experimental and holding temperatures were seasonally dependent. Most of the experiments were done during summer months and continued into late fall as the sea-water temperature at the laboratory intake warmed from 14 to 22° C (August) and fell again to about 12° C (December). In all cases, the experiments were done either at the intake temperature or at higher temperatures maintained by mixing of "raw" intake water and warmed sea water from the large reservoir in the recirculating system of the aquarium. During holding periods the fish were fed daily on diced herring and soft-shell clams.

Since completion of studies at Woods Hole for species listed in Table I, preliminary results to be reported elsewhere have been obtained using similar procedures. These results are included in Table III (Roberts, unpublished) and in the discussion. The report on the sand tiger shark given in Table III was based on repeated visual inspection of a single specimen swimming in the large display tank at the aquarium at Woods Hole. Remarks on non-ventilating rockfish apply to the blue rockfish, *Sebastes mystinus*, the olive rockfish, *Sebastes serranoides*, and the bocaccio, *Sebastes paucispinis* taken at Tanner Bank near San Clemente Island, California.

Activity recording during swimming

Swimming apparatus. The fish were made to swim against a pump stream in a version of the swimming chamber used by Sutterlin (1969) in order to determine critical swimming velocities for transitions from active to passive gill ven-

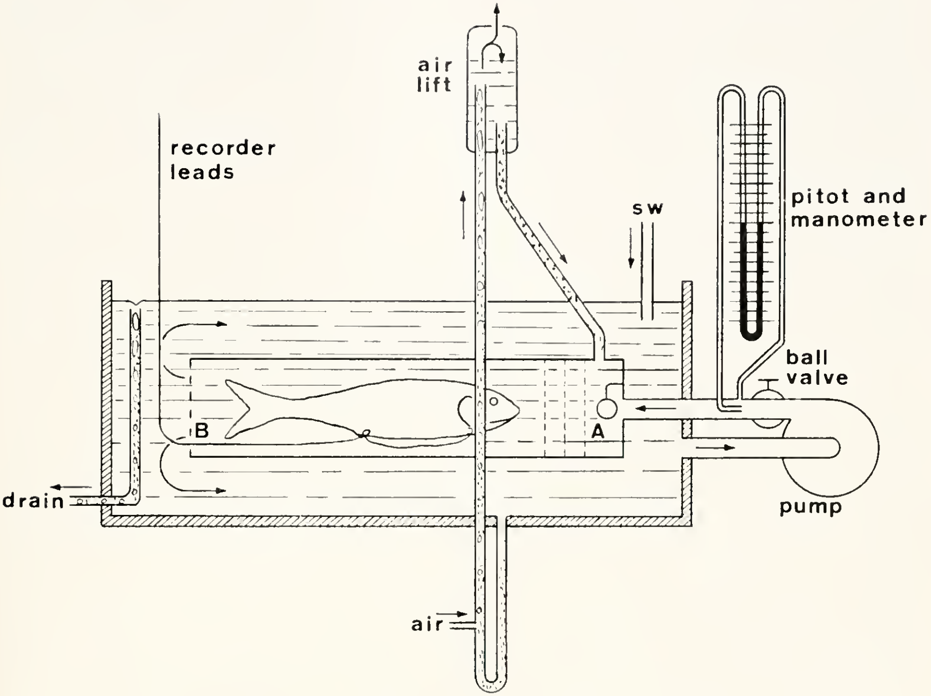


FIGURE 1. Diagrammatic representation of the swimming tunnel used. Design features are similar to those of Sutterlin (1967) with the addition of the air lift circulator for maintaining a slow velocity flow through the tunnel, and fitting around the outer chamber of a 1/4 inch-mesh galvanized Faraday cage. The symbols *A* and *B* represent respectively, the front ball spreader and flow screens, and the rear retainer screen (see text). Temperature control was obtained by use of a thermoregulator-heater located in the outer bath and operated at a set point higher than the entering sea water (*sw*).

tilation (Fig. 1). The centrifugal pump (neoprene body and impeller) of 1/3 hp produced flow velocities up to 90 $cm \cdot sec^{-1}$ in the plexiglas swimming tube (78.5 mm ID) by control of a PVC ball-valve located between the pump outlet and the pitot flow meter. Microturbulent flow and a uniform front were maintained by a ball "spreader" and three, 16-mesh stainless-steel screens at the front of the swimming tube. The rear end of the tube was fitted with a coarser screen of 6-mesh to confine the fish within the tube and to permit free movement of the electrode cable trailing from the fish as it changed its position in response to changes in flow velocity.

Calibration of flow velocity. The pitot flow-meter was calibrated indirectly because the flow relationships between the inflow tube from the pump and the swimming chamber proved to be non-linear largely due to back-pressure effects of the spreader ball and the three front screens. Two methods were used that gave similar flow rates for given deflections of the mercury manometer (tubing spaces, water filled). For the first method, slugs of methylene blue dye injected into the pump intake were timed as they passed between the last flow-control

ARY SS. ATORY

screen at the front, and the rear screen of the swimming tube. The second method (Sutterlin, 1969) was used with the bath filled with fresh water and depended upon timing conductance changes in the stream flow between the electrode pairs located in the walls of the swimming tube, front and rear, as small amounts of saturated KCl, injected into the pump intake, passed the electrodes. Corrections for fish displacement were not made for the cross-sectional area of the fish seldom exceeded 1/10 of the swimming tube.

Electrode placement and activity waveforms. Each fish was anesthetized in MS 222 solution (0.1 $g \cdot l^{-1}$) for insertion of electrode pairs for recording waveforms of muscle action potentials related to respiratory and cardiac cycles (Roberts, 1964; Sutterlin, 1969; Spoor, Neiheisel and Drummond, 1971). Electrode pairs were made up of lapwound, 1-m lengths of 45 gauge, insulated stainless-steel wire (Johnson, Matthey and Co., Ltd., England, annealed, epoxy coated) with bared and hooked tips threaded through 22 gauge syringe needles. These were inserted, one pair into the pericardial space, and the other into one of the ventilatory muscles, usually the *adductor mandibularis*. The needles were withdrawn after electrode placement, and the wires secured by skin ties at the insertion site and again at the anal opening. In this way, looping of the formed cable around the tail and tangling was minimized after recovery of the fish in the swimming tube. When tail-beat frequencies were to be recorded (*e.g.*, mullet), *mu*-metal bands (high magnetic permeability) were wrapped around the caudal peduncle for proximity detection of lateral tail movements. A detector coil with permanent field magnets was fitted around the outside of the swimming tube for this purpose. It was made up by winding 38 gauge enameled magnet wire around a coil form incorporating eight Alnico bar magnets (6 mm by 48 mm long, spaced 20 mm; 4 each side). The magnets were oriented parallel to the tube axis with like polarity (north to north) on each side, but opposite in polarity between the two sides, in order to maximize the horizontal magnetic flux density across the tube. The finished coil form was potted in epoxy resin to form a loose fitting, short cylinder. The lead cable incorporated a two-stage RC filter to reduce 60 cycle interference. Distortion of the field by the moving tail band of *mu*-metal generated an AC signal at the tail-beat frequency adequate for polygraph recording at a gain level of 10 to 50 $\mu V \cdot mm^{-1}$.

Usually it was possible to record waveform of both cardiac and ventilatory cycles on a single channel of the R-series Dynographs used (Beckman Instruments, Inc.). This was done by trial-and-error combination of the four electrode leads in the trailing cable, and by appropriate adjustments of the band-pass filters on the input couplers of the polygraph. When clear differentiation of the two waveforms was not possible on one channel, or when tail-beat frequencies were desired, two recording channels were used (*e.g.*, Fig. 4, mackerel and mullet). Usually, the high frequency components of the EMG (electromyogram) signals were suppressed by band-pass control so that only the slow wave part of the signal was displayed by the pen writer. This was done usually to enable differentiation of ECG (electrocardiogram) and EMG waveforms on a single record channel.

Training and experimental procedures

One to several hours allowed recovery from anesthesia once the fish were put into the swimming tube with the air-lift circulation on. Additional time was required to train fish to swim steadily in the pump stream flow as velocities were changed. The most effective training method was to just slightly open the control valve with the pump running so that the fish drifted to the rear screen. Within 5 to 10 minutes, the fish would begin slow swimming to avoid tail flexion against the screen. Flow velocity was then slowly increased until the fish again stopped or began dart swimming or thrashing. When this happened, the pump was shut off and the animal given a brief rest lasting 5 to 10 minutes before another training period. Training was considered complete when the animal would maintain a relatively fixed position between front and back screens as the control valve was turned from off to full on and the reverse.

Once a fish was trained to sustain swimming over the complete velocity range of the apparatus, a routine procedure was followed. A rest period of about 10 minutes was allowed after each swimming bout. Transition velocities for ram ventilation were established in most cases by four swimming periods. The pump was turned on after a rest period and the control valve opened in stages to give flow velocities that approximated step increases of 5 to 10 $\text{cm}\cdot\text{sec}^{-1}$ flow velocity (manometer pressure equivalent) until the velocity maximum for the pump was reached. Sufficient time was allowed at each step to allow the manometer to stabilize and to obtain a 15 second polygraph record of cardiac and opercular cycles. The entire range of possible flow velocities with the fish swimming was covered in this way in about 10 minutes. Following the rest period, the sequence was repeated in reverse order. The reverse sequence was begun with the pump full on and then followed by step-wise reductions in pump flow until the pump was fully throttled, and the fish again at rest. One repeat usually sufficed unless excessive electronic noise or weak muscle signals made derivation of event rates from the polygraph record difficult.

RESULTS

Ram ventilation

Critical swimming velocity. The transition to ram gill ventilation in fish is a graded process as swimming picks up from rest. The first indication that a critical swimming speed has been reached is signalled by the drop-out of single cycles. The drop-out continues until only occasional ventilatory movements and "coughs" are noticed. A graded series just following the training period for a bluefish is illustrated in Figure 2. Return of active movements with gradual reductions in swimming velocity to below critical shows nearly the same sequence, but in reverse order. Generally, subsequent swimming bouts with this and the other ram-ventilating species showed some decrease in the velocity critical for the transition to ram ventilation. In fact, transition speeds of experienced fish may fall to less than half that observed in initial tests so that switch-over to ram ventilation can occur at a surprisingly slow swimming speed. This contrast is best noted by comparison of the bluefish swimming bout given as Figure 2 and the transition range for all bluefish tested summarized in Table II.

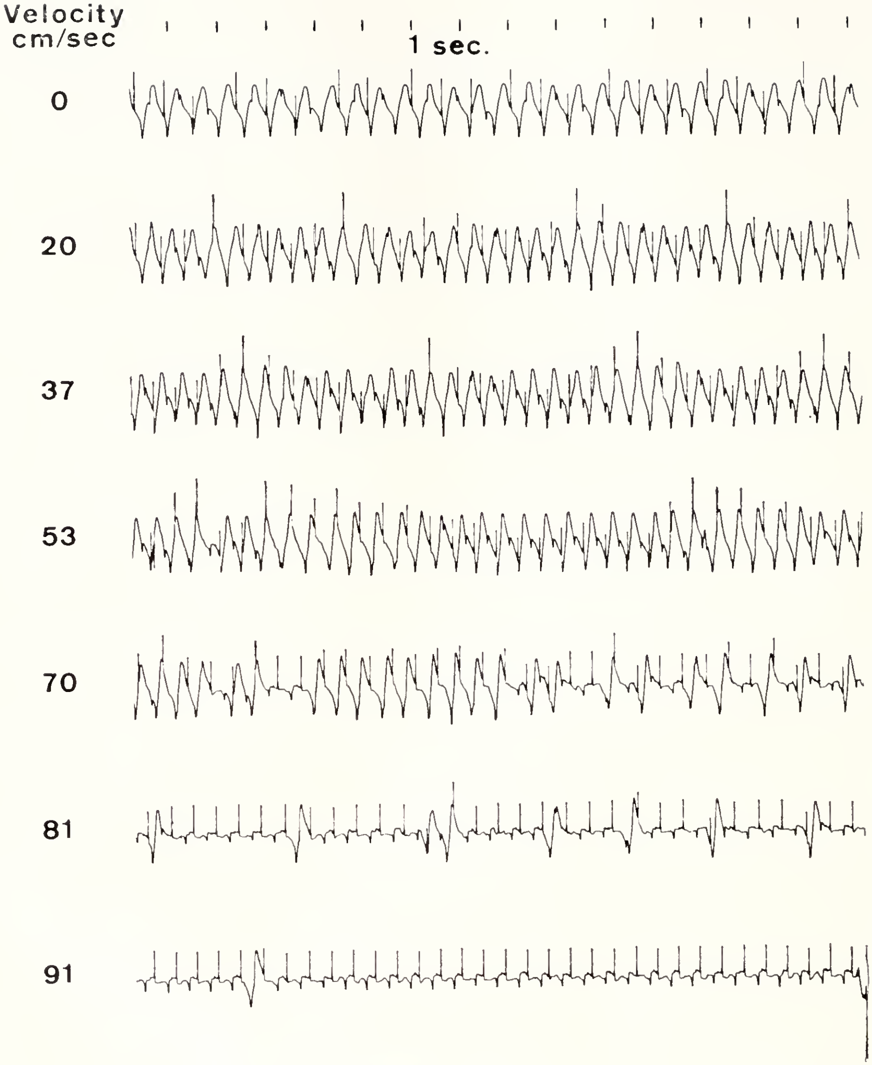


FIGURE 2. A combined sequence of EMG slow-wave components recorded from the *adductor mandibularis* muscle (left side) and the ECG of a bluefish during acceleration to and above a swimming speed sufficient to support ram gill ventilation. The time marks in this and subsequent figures denote 1-second intervals.

Although patterns for conversion to ram gill ventilation of different fish are similar, there does seem to be great variation between individuals in their behavioral selection of swimming velocities for switching ventilation modes. Figure 3 shows conversion patterns for each of the five mackerel used in the study (see also Heath, 1973). Clearly, the transition can be gradual or abrupt, and with or without a marked increase in the frequency of active respiratory movements prior to adoption of the ram mode.

TABLE II

Mean values and ranges for opercular (O) and cardiac (C) rate changes in resting and active marine fishes

Species (N)		Aquarium display cpm	Resting rate swimming tube cpm	Active rate at transition velocity cpm	Active rate at 90 cm ·sec^{-1}	% change from rest		Swimming speed at transition cm·sec^{-1}
						At transition	At 90cm ·sec^{-1}	
Mackerel (5)	O	—	124 (110–140)	134 (125–175)	—	8	—	67 (53–75)
	C	—	100 (72–133)	121 (89–140)	126 (98–140)	21	26	
Blue runner (7)	O	—	80 (52–105)	119 (90–135)	—	49	—	48 (35–75)
	C	—	43 (26– 62)	62 (50– 92)	72 (57–105)	44	67	
Bluefish (11)	O	77 (SD, ±13)	99 (65–145)	138 (105–185)	—	39	—	69 (49–82)
	C	—	83 (52–115)	113 (92–160)	117 (98–160)	36	41	
Northern scup (5)	O	51 (SD, ±10)	148 (120–180)	196 (175–200)	—	32	—	66 (44–79)
	C	—	81 (54–105)	142 (80–150)	146 (130–160)	75	80	
Mullet (4)	O	—	148 (113–180)	—	201 (130–232)	—	36	—
	C	—	104 (88–120)	—	124 (112–132)	—	19	

Conversions to ram ventilation, the mullet excepted, were completed at velocities under 90 cm·sec^{-1} by all the fish tested. This is a swimming-speed equivalent of 2.7 to 4.7 BL·sec^{-1} (body lengths per second) according to the size of the mackerel used (Figs. 3 and 4). This range does not substantially exceed the open-sea cruising speed expected for mackerel of this size or roughly 2 BL·sec^{-1}, as based on Magnuson's estimates for speed minimums required to maintain hydrostatic equilibrium (1970). Table II gives similar ranges and mean values for complete adoption of ram ventilation by all of the species groups studied.

Individuals of all species tested occasionally stopped swimming at flow velocities that were above critical and drifted to the rear screen of the chamber. When this happened, ventilation movements most often reappeared immediately and continued until swimming was resumed. Two of the blue runners continued ram ventilation in several swimming bouts after drifting back to the rear screen. This unusual tactic was not observed in swimming bouts with the other marine species. Note also (Table II) that the average transition velocity for blue runners seems to be lower than for the other ram ventilators. A notable exception to be considered later, is the remora which ram ventilates attached to its swimming host, but only above velocities of about 1 km (Muir and Buckley, 1967).

None of the mullet showed signs of even partial conversion to ram ventilation during enforced swimming, despite the fact that they trained readily to steady swimming in the apparatus. These fish appeared to be in excellent health, but had all been captured and held in the aquarium at Woods Hole about 1 month prior to their use. All survived the swimming bouts for at least a month after electrode removal in one of the holding tanks. Figure 4 includes a typical record

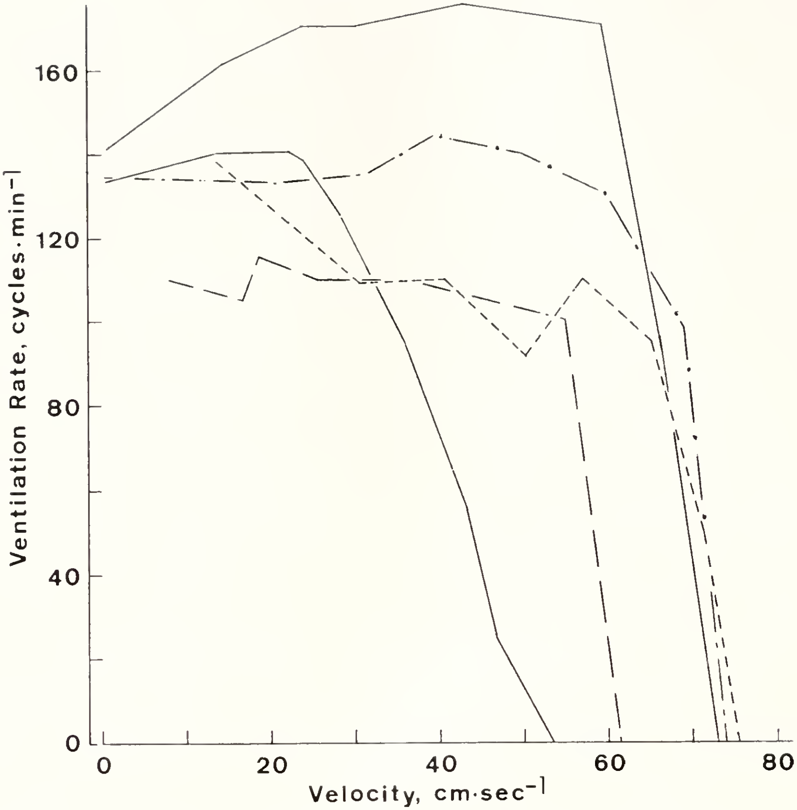


FIGURE 3. Active gill ventilation rates of five Atlantic mackerel (see Tables I and II) relative to enforced swimming speed. Rates were determined by counting all cyclic ventilatory movements that appeared on 30-second strips of polygraph record.

with a simultaneous display of tail beats obtained by proximity detection as the pump flow was valved from off to full-on to induce swimming at 85 to 90 cm·sec^{-1}. The records shown for the other species were obtained in a similar way by rapid valving of the pump output to produce swimming at speeds below and above the active to passive transitions in ventilation.

Transition characteristics. Figure 4 also demonstrates that timing differences are consistent between the onset of ram ventilation and return to active ventilation when swimming speeds are abruptly increased or decreased by manipulation of the pump flow. The return to active ventilation by most of the fish used generally was found to require less time than switching to the ram mode. Figure 5 contrasts in that conversions between ventilatory modes by this bluefish proved to be nearly equal in timing. The second, early return to active ventilation (Fig. 5, down arrow) most likely represents a single movement, seen to occur normally in some swimming bouts (Fig. 2, 91 cm·sec^{-1}). Allowing for the chance occurrence of single respiratory movements and possible operational errors, a great

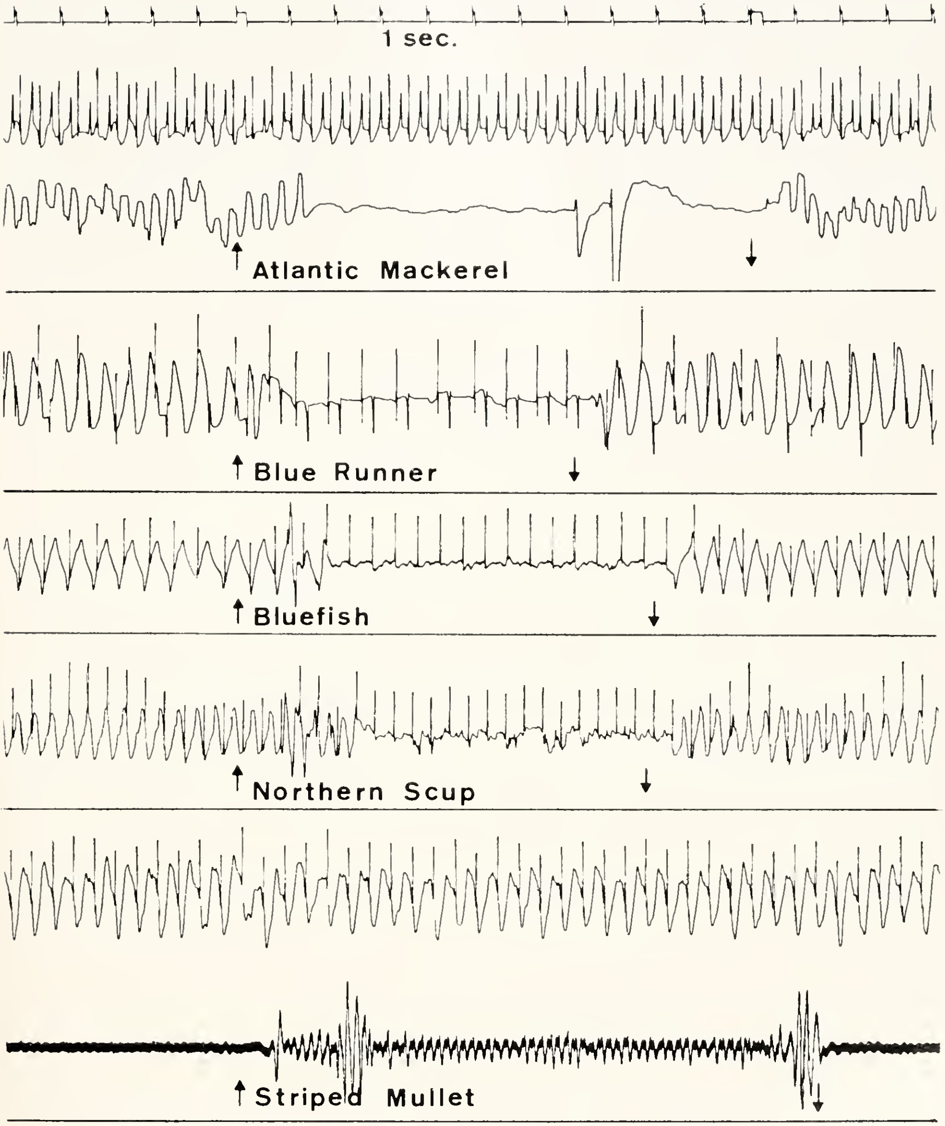


FIGURE 4. Active to passive transitions in gill ventilation of five marine species from rest to swimming just above transition velocities following turn-on (arrow up) and turn-off (arrow down) of the pump. The lower trace for the Atlantic mackerel is shown for clarity in separation of cardiac and ventilatory events. The lower record for the striped mullet represents tail-beat frequency recorded by proximity detection of the movement of *mu*-metal bands around the caudal fin peduncle. Changes in heart rates are slight due to the lack of rest periods prior to these sequences.

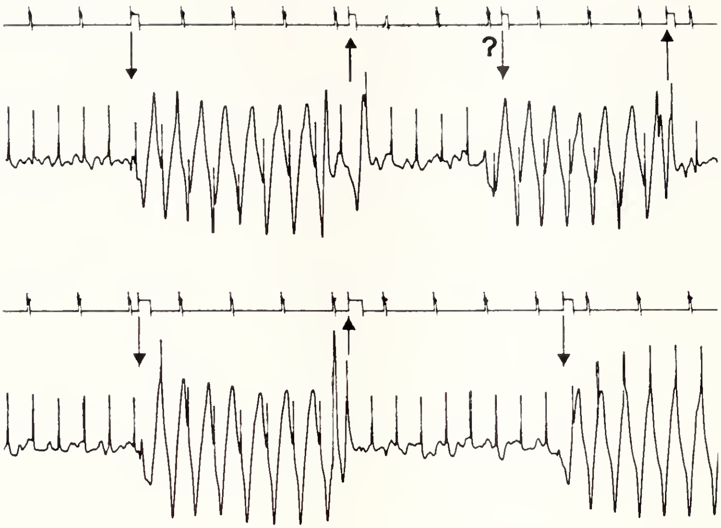


FIGURE 5. Repeated sequences of pump valving to vary swimming speeds of a bluefish from just above to just below velocities for conversion between active and passive modes of gill ventilation. Up and down arrows indicate respectively, decreasing and increasing pump flow.

number of switching-time measurements indicate that all of the ram ventilators can initiate active ventilation in as short a period as 0.2 second following sudden drops in the speed of water flow to below critical swimming rates for ram ventilation.

Usually the transition to the ram mode was marked by completion of at least one complete ventilatory cycle before actual ram gill ventilation began. Strong "coughs" were sometimes seen, both at the onset of ram ventilation, and preceding the return of rhythmic breathing, as swimming velocities were made to change above and below critical speeds for switching between modes. In other respects, the pattern of single ventilatory movements shows little change in amplitude or timing as swimming begins and the supression of individual cycles occur with ram gill ventilation (Fig. 2).

Frequency changes—branchial and cardiac pumps

Gill ventilation rates of wired fish recorded before and after swimming bouts (30 minutes rest) all greatly exceed rates determined for inactive, undisturbed fish. For example, Table II lists rates for experimental bluefish and scup as well as rates for display fish at the aquarium at Woods Hole. The latter were recorded at 9 a.m. before public open hours (Logan and Roberts, unpublished observations; Roberts, 1974). Large differences such as these can be expected because handling fish elevates their metabolism and excitability for prolonged periods (Roberts, 1964; Muir and Buckley, 1967; Sutterlin, 1969; Spoor, Neiheisel and Drummond, 1971). The same can be said for heart-beat rates although these were not recorded along with opercular frequencies of the display bluefish and scup.

Table II also shows that heart and ventilatory rates increase proportionally with swimming velocity, although variability is large. Beyond the transition speed for conversion to ram ventilation, heart rates of all species increased with further increase in swimming speed. No obvious cardiac rate changes or alterations in ECG patterns were found associated with conversions between active and passive gill ventilation (Figs. 2, 4, and 5). Even at the higher swimming velocities, visual stimuli (shadows, movements of the operator) produced the well-known reflex bradycardia (Labat, 1966; Randall, 1970a; Roberts, 1973). Therefore, considerable reserve accommodation in cardiac function remains at moderate swimming velocities, and at speeds higher than necessary to support ram ventilation.

Discussion

In a sense it is surprising that the use of ram gill ventilation is widespread among fishes other than those known to be high-speed or continuous swimmers of open-water habit. Yet, if the supposition is correct that ventilatory efficiency improves with conversion from active to passive ventilation once swimming velocity reaches 1.5 km sec^{-1} (Brown and Muir, 1970) the advantage of conversion between stops for feeding or other fish activity becomes more obvious (Roberts, 1974).

There is a proviso, however. That is, successful ram ventilation seems to require that a fish be above a certain size so its normal cruising speed exceeds the velocity minimum for support of the passive ventilation mode. For example, it was mentioned above that scombrids have outgrown the capacity of their active branchial pump system so adult survival is dependent upon continuous swimming for a variety of reasons (Hall, 1930; Brown and Muir, 1970; Magnuson, 1970, 1973). Apparently the Atlantic mackerel, and most scombrids as well, lose the ability to ventilate the gills by rhythmic breathing as an ontogenetic development. Captive juvenile Atlantic mackerel ranging in body size from 2 to 12 cm actively ventilate their gills and often "stand dead in the water" (personal observations, aquarium, Woods Hole). Most likely, fish this small simply are not able to swim continuously at speeds fast enough to sustain ram ventilation.

Table III lists species known to utilize ram ventilation. It includes only two species from among the sharks and omits many probable ram ventilators from both major classes of fish only for the reason that many reports lack reasonable substantiation. The list is based upon careful visual, physiological and cinemaphotographic monitoring. Brief comments on habitats were abstracted from the monographs of Bigelow and Schroeder (1953) and Miller and Lea (1972) for the coastal marine fishes of the Atlantic northeast and California, respectively. Common names and ordering of the families and species have followed the recommendations of the Committee on Names of Fishes, American Fisheries Society (1970).

Just as important is the fact that some fish do not ram ventilate at all. The mullet is a curious exception for it is an inshore, near surface high-speed swimmer. It has an unusually small mouth and feeds mostly on plankton and algae. Perhaps the mullet's active respiratory mode is somehow linked with its feeding style during swimming.

Demersal fish, notably those equipped with large branchiostegal baskets (see McAllister, 1968) probably do not convert to passive gill ventilation at any swim-

TABLE III

Ram ventilating fish

Species and systematic position	Habitat	Method	Source
Chondrichthyes			
Odontaspididae			
Odontaspis taurus (sand tiger)	subtidal, beaches and bays	visual count, aquarium	Roberts (unpublished) von Wahlert (1964)
Carcharhinidae			
Triakis semifasciata (leopard shark)	subtidal, beaches and bays	cinema photography, aquarium	Hughes (1960)
Osteichthyes			
Salmonidae			
Onchorhynchus nerka (*sockeye salmon*)	pelagic and mid-water, anadromous	branchial pressures	Smith *et al* (1967)
Salmo gairdneri (rainbow trout)	fresh waters and anadromous	electromyograms	Roberts (unpublished)
Percichthyidae			
Morone saxatilis (striped bass)	near shore pelagic and fresh waters, anadromous	electromyograms	Roberts (this paper)
Echeneidae			
Remora remora (remora)	open ocean, surface to mid-water	cinema photography, water tunnel	Muir and Buckley (1967)
Pomatomidae			
Pomatomus saltatrix (bluefish)	near shore and bays, pelagic to mid-water	electromyograms	Roberts (this paper)
Carangidae			
Caranx crysos (blue runner)	near shore and bays, pelagic to mid-water	electromyograms	Roberts (this paper)
Trachurus symmetricus (jack mackerel)	near shore to open ocean, pelagic to mid-water	electromyograms	Roberts (unpublished)
Sparidae			
Stenotomus chrysops (scup)	demersal to mid-water	electromyograms	Roberts (this paper)
Kyphosidae			
Medialuna californiensis (halfmoon)	mid-water, kelp beds	electromyograms	Roberts (unpublished)
Scombridae			
Katsuwonus pelamis (skipjack tuna)	pelagic, open ocean	cinema photography (NMFS, Honolulu)	Brown and Muir (1970)
Scomber japonicus (Pacific mackerel)	near shore, pelagic	electromyograms	Roberts (unpublished)
Scomber scombrus (Atlantic mackerel)	near shore, pelagic	electromyograms	Roberts (this paper)

ming velocity. Their respiratory apparatus is well designed for respiration in standing water and functionally, the opercular pump dominates. About 90% of the respiratory cycle at rest is spent in slow opercular aspiration of water through the gills with the mouth open (Hughes, 1960a; Hughes and Roberts, 1969; Roberts, 1974). Consequently, respiration rates of these fish usually are considerably lower than their cardiac rates (Hughes, personal communication; Roberts, 1974). In fact, comparison of resting ventilation and cardiac frequencies serves as a "rule of thumb" to differentiate between fish that depend primarily upon the action of the opercular pump and fish in which branchial ventilation is equally shared by the buccal and opercular pumps. For these, rate ratios of about one can be expected. The latter pattern for propelling water across the gills generally prevails among fast swimmers of mid-water and pelagic habits. The fast swimmers usually are good ram ventilators as well.

For an example, the family Scorpaenidae can be cited. The family includes two major groups, the scorpionfish and the rockfish, *Sebastes*. All have well-developed branchiostegal rays and membranes. A number of the rockfish have evolved away from a strictly demersal existence. Some closely resemble the sea basses (Serranidae) of the genus *Paralabrax* in body form, development of the branchiostegal system and habits. None of these fish types so far examined ram ventilate (*P. clathratus*; *S. mystinus, serranoides, paucispinis*). Yet, with the exception of the blue rockfish, *S. mystinus*, all trained rapidly to enforced swimming at speeds well above the requirement for sustained ram ventilation.

Another fish, the anadromous striped bass, *Morone saxatilis* (Percichthyidae), sometimes included with the Serranidae (Miller and Lea, 1972), seems ready to adopt the ram mode of gill ventilation at a swimming speed of about 50 $cm \cdot sec^{-1}$. Only a single specimen was tested (total length, 46 cm). Unlike the serranids, however, the branchiostegals of the striped bass are much reduced and the cardiac-ventilatory rate ratio is about one.

Just as extensive development of the branchiostegal system facilitates respiration in quiet water, this development probably sets a limit to the speed at which fish can cruise routinely and still ensure reasonable energy expenditures for gill ventilation. Structural stability of the lightly-build branchiostegal rays and membranes, and the drag resistance they present when laterally expanded during swimming are important considerations. But as yet, no detailed hydrodynamic analyses exist to describe the array of niche compromises made during the evolution of modern fishes to meet the joint needs of respiratory gas exchange, and swimming for prey capture, migration, and reproduction.

Experimental verification that many swimming fish ram ventilate has been a relatively simple task. Providing the answers to why and how ram gill ventilation occurs has been more difficult. Of the two questions, the answer to why is the hardest to supply in satisfactory form at the present stage of investigations on fish energetics. A large part of the problem rests with assessing the efficiency of gill ventilation in swimming fish and hence, the work load of ventilation with respect to total body metabolism (see also Jones, 1971).

Estimates of energetic costs of active gill ventilation versus total metabolism in resting fish range from a low of 0.5% (Alexander, 1970) to as high as 43% (Schumann and Piiper, 1966). Jones calculations for a trout model (1971), reveal a theoretical cost as small as 1%, despite assumption of a low resting efficiency for the operation of the branchial musculature in propelling water through the gills (3 to 4% efficiency). Intermediate cost estimates of 10% (Hughes, 1973) and 5 to 15% (Cameron and Cech, 1970) also have been suggested. However, the general accuracy of these estimates as applied to rhythmic gill ventilation is doubtful due to the complex hydrodynamic integration of changing flow rates and pressure profiles that occurs during single respiratory cycles (Hughes and Saunders, 1970; Ballintijn, 1972). The critical factors of environmental temperature and oxygen availability also must be considered for both exert strong influences upon ventilatory stroke volume, coupling of the branchial pumps, and hence upon respiratory efficiency (Hughes and Roberts, 1970; Hughes and Saunders, 1970; Heath and Hughes, 1973).

Respiratory energetics of sluggish demersal species that have elaborate branchiostegal systems scarcely are comparable with the energetics of pelagic, high-speed swimmers which have only remnants of branchiostegal rays and membranes. The energy cost of active ventilation for a bottom-living fish probably is high, but affordable. They often are opportunistic predators that swim in short darts to seize prey or swim slowly to feed on other sluggish bottom species. Midwater and pelagic fish contrast for they usually depend upon chasing down active prey or upon planktonic gathering and are more or less continuous swimmers. Their metabolic costs for gill ventilation also may be high during brief pauses in swimming, but the cost probably does not increase proportionately with total metabolism during swimming up to ram-ventilating speeds. The reason is that when a fish begins to swim, its forward motion causes pressure to rise at the mouth, partially relieving power requirements for the buccal fore-pump (Hughes, 1960a; Hughes and Shelton, 1962; Cameron and Cech, 1970). Opercular aspiration also may be facilitated by the venturi effect as water streaming along the body passes the gill-cover margins in spite of some drag losses due to cyclic abductions of the opercular doors (also see Brown and Muir, 1970).

Further acceleration to a ram speed results in a graded transfer of the metabolic cost of rhythmic ventilatory movements to the drag forces of swimming (Brown and Muir, 1970). Some lesser amount of energy must be reserved for tonic holding of the mouth gape and the opercular exit slots to sizes suitable for respiratory needs once the conversion to ram gill ventilation is complete. Thus, the switchover from active to passive gill ventilation is a tactic that probably saves considerable energy. However, not all of the savings result simply from the transfer of respiratory work from one set of muscles to another. Loss in locomotory efficiency is also a probable consequence of rhythmic gill ventilation, especially at high speeds. For example, cyclic variations occur in the cross-sectional area of the head as the opercular doors open and close. Changes in mouth gape also occur with operation of the buccal phase of branchial pumping. Such drag oscillations are likely as well to generate periodic turbulence, adding still further to the work load of swimming. Little is known about these factors and their effects on swimming. But, the available evidence shows that the events of gill ventilation and of swimming must be tightly coupled by unavoidable hydrodynamic interactions. The same should be true of their relative metabolic efficiencies.

Only a single study has been directed toward assessment of the work load during ram gill ventilation. Brown and Muir (1970) have calculated the ventilation drag-resistance of a 44-cm skipjack tuna (*K. pelamis*) swimming at a basal speed of 66 cm·sec^{-1} to be about 7% of the total body drag. Translation of the drag forces into metabolic expenditures for swimming and other functions, has led them to estimate the cost of ram ventilation for a tuna swimming at basal speed to range from 1 to 3% of total metabolism—a cost much lower than most prior estimates for active ventilation in non-swimming fish.

Aside from the fact that ram gill ventilation is used by most active fish that have been tested (Table III), one unusual example also attests to the efficacy of the ram mode. The small shark sucker (*R. remora*) normally is a sluggish swimmer that uses active ventilation. Yet when this fish attaches to a passing shark or other large swimming host, it too can adopt the ram mode when the host reaches the requisite speed. In a sense, they gain a "free ride" by transferring the work load of gill ventilation to their hosts. But, they seem to do it by using neural adaptations for ram ventilation that most likely are ancestral to the adaptations which permit their form of locomotory and feeding commensalism (Table III). Muir and Buckley (1967) have found that remoras placed in a water tunnel would attach to the wall as flow velocities through the tunnel reached 20 to 30 cm·sec^{-1}. Switch-over from active to passive gill ventilation was found to range from 50 to 80 cm·sec^{-1}. This is a range remarkably similar to the transition velocities reported here for other marine species (Table II).

The work expended by a species in ventilating its gills between a dead stop and a burst of maximal swimming must represent a compromise with costs of other activities as defined by its niche and physical factors in its habitat. Ultimately, as Brett has shown (1964) for the sockeye salmon (*O. nerka*), the maximal sustainable swimming speed of a fish will be limited by the cost of gill ventilation, and its ability to manage an oxygen debt, combined with the costs of cardiac pumping as Jones suggests (1971). This means that routine levels of activity in swimming, feeding, and ventilating the gills will represent partitioning of energy available for these functions according to a fish's life style whether it is benthic or pelagic, and whether it is a sluggish swimmer or a very active one. If Jones' (1971) and Brown and Muir's (1970) attempts to model ventilatory energetics are reasonable, the costs of gill ventilation for active fish that ram ventilate may remain as low as 1 to 3% of total metabolism over a wide range of swimming speeds.

What speed might mark an upper limit for a good swimmer is difficult to estimate, but it is probably not less than cruising velocities for most pelagic species. Very likely another factor becomes significant at still higher speeds or during burst swimming. A fall in the %-utilization of oxygen from the respiratory stream should occur as the ventilatory minute-volume increases with increasing metabolic needs (Hughes, 1966; Randall, 1970b; Hughes and Morgan, 1973). This represents a loss in respiratory efficiency that would be additive to the work load of swimming.

Much could be learned to substantiate their trout and skipjack estimates by use of water or wind tunnel models. In fact, the brancial system of fish respiring in the ram mode resembles, in an engineering sense, the design of a combined

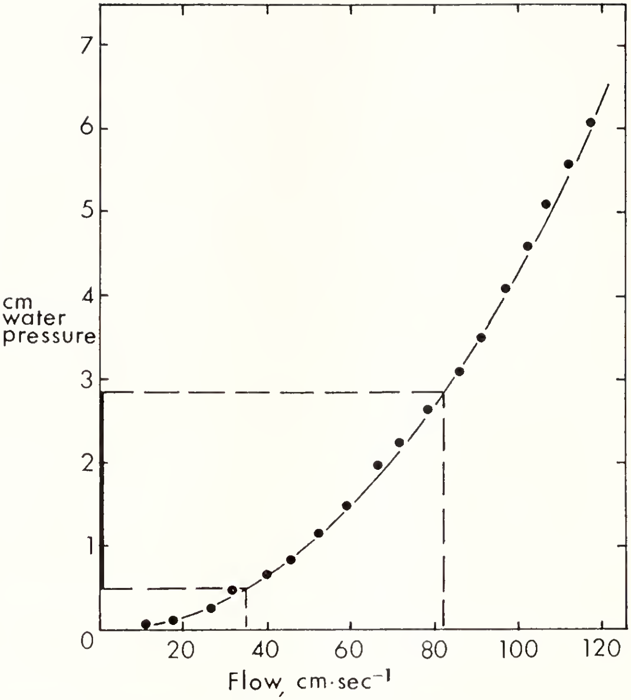


FIGURE 6. Secondary calibration of a Blažka swimming tunnel (respirometer type) with a combined impact-reverse pitot tube in the axial flow of the swimming chamber to give pressure equivalents of flow velocity in centimeters of water. The primary calibration was obtained by use of a ducted, impeller flow-meter (Marine Advisers Inc., La Jolla, California; model B-7C). Minimal and maximal estimates for across-gill differential pressures have been extrapolated on the ordinate according to the range of swimming speeds permitting ram gill ventilation for the fishes listed in Table II.

impact-reverse pitot tube with variable up- and downstream orifices. The comparison has limits. The pitot tube typically is a zero-flow pressure measuring device so the resistance between orifices is infinite. The branchial system differs in that through-flow occurs as a volume rate determined primarily by the resistivity of the gill screen. Figure 6 illustrates the pressure differential developed between impact and reverse openings of a pitot tube located in a "swimming" respirometer of the Blažka type (loaned by Dr. F. E. J. Fry, University of Toronto) relative to water flow velocities from 0 to 120 cm·sec^{-1}. The extrapolated buccal-post branchial pressure range also is shown (ordinate). This range of pressure differences corresponds to minimal and maximal swimming speeds for ram gill ventilation by the fish species listed in Table II (*i.e.*, blue runner and bluefish, respectively). Assuming that a resemblance to the combined impact-reverse pitot is reasonable, then an across-gill pressure drop as low as 0.5 cm enables some fish to swim as slowly as 35 cm·sec^{-1} and still support ram gill ventilation.

Muir and Buckley (1967) have determined via buccal and opercular cannulas that the average pressure necessary to force water through the gills of actively breathing remoras is 0.87 cm. Yet, these investigators have estimated that the passive mode of ventilation in swimming remoras requires an across-gill pressure drop of 1.87 cm water pressure. The latter figure probably is too high for a minimal value for it was based on the use of a simple impact pitot that does not correct for static pressures generated in the positive-pressure pumping system they employed. However, if the minimal swimming speed for conversion to ram ventilation by a remora is selected (page 583, 50 $cm \cdot sec^{-1}$, Muir and Buckley, 1967), and the curve in Figure 6 is used, a differential of just over 1 cm water pressure results. Therefore, when an allowance is made for inertial losses that must occur with branchial flow-rate changes during active as opposed to passive gill ventilation, the suggested minimal value of a 0.5 cm differential pressure to support ram gill ventilation seems acceptable.

The transition to ram ventilation from active gill ventilation in swimming teleosts superfically resembles breath holding in man and diving aerial-breathers among vertebrates. It is marked by cessation of rhythmic breathing and seems not to be mediated by chemoreceptive detection of changes in the concentration of respiratory gases or pH. Recent experiments to be reported elsewhere, indicate that the transition swimming velocity for ram ventilation is only slightly increased by warming (1° C per minute; bluefish, blue runners) or by dropping the pO_2 in a swimming tunnel (halfmoons, jack mackerel), and then only at extremes.

Active ventilation in resting fish is driven by the combined actions of the buccal and opercular pumps; effected by contractions in antagonistic arrangements of the branchial musculature (Ballintijn and Hughes, 1965; Ballintijn, 1972). Pressure generated by streaming flow at the mouth allows transfer of much of the ventilatory work to the swimming musculature as the transition swimming speed for ram ventilation modes is accomplished by reflex conversion of motor outputs from the medullary respiratory areas of the CNS from a cyclic to a tonic drive of the branchial muscles.

The fact that tonic control of the buccal musculature is maintained during ram gill ventilation has been verified for a mackerel by Brown and Muir (1970). They found that progressive reductions in the pO_2 of water circulating in a water-tunnel respirometer results in graded increases in mouth gape at fixed swimming speeds. Furthermore, the mouth of an anesthetized fish usually is forced fully open or fully closed in the stream flow of a swimming tunnel, depending upon the streamline shape of the head and the degree of mouth gape when the flow is started. Clearly, mouth gape opening is not a passive process in swimming fish that do ram ventilate.

Despite the large effort that has been spent on the examination of adaptive respiratory reflexes in fish, specific receptors for initiating reflex action have not been identified. However, it is known that proprioceptive elements sensitive to gill filament displacement are located in the gill arches (Sutterlin and Saunders, 1969). Pertinent to this discussion are receptors for monitoring water flow velocity, and muscular activity effecting ventilation and swimming.

Delay in the return of active gill ventilation is so short in a ram ventilating fish when swimming slows or stops, that reflex switching by way of oxygen sampling is unlikely for this event (Figs. 4, 5). Return of rhythmic breathing in bluefish and blue runners can occur within 0.3 second of flow shut-down in a swimming tunnel. Timing in the blocking of rhythmic movements as swimming begins at speeds above the transition speed for ram gill ventilation is a different case. Several seconds may elapse before a fish begins to ram ventilate, but during this time there will not have been any interruption of the branchial water flow.

Receptor systems subject only to minor influences by metabolic loading or by temperature change would seem to provide the most reliable cues for switching between ventilatory modes. Mechanoreceptors sensitive to water flow velocity such as elements of the acoustico-lateralis system, and proprioceptors in the gill arches and the swimming musculature (stretch receptors) are likely candidates for this function. Monitoring of water flow velocity over the body and through the branchial chambers, and the detection of tail-beat frequency should be influenced only by the properties of water as a flowing fluid (see Hughes and Roberts, 1970; Randall, 1970b). Consequently, the thermal error in receptor functioning should be negligible for fish swimming at set velocities over the temperature range of their usual habitats.

Although both receptor detection of water-flow rates and events in swimming may be critical for reflex conversion to ram gill ventilation as reported above, some exceptions have been noted. Two of the blue runners from the group listed in Table II were found to continue ram gill ventilation after drifting to the rear screen of the swimming apparatus during swimming bouts. No obvious signs of fatigue were observed when they quit swimming. Under the unnatural circumstances of forced holding of these fish in a stream flow, it is possible that conditioning occurred that permitted continuation of the ram mode. A similar explanation might be applied to the ram gill ventilation of remoras attached to the wall of a swimming tunnel. However, Muir and Buckley (1967, page 582) also have reported that the swimming musculature becomes relaxed when the flow reaches ram ventilating speeds so that the body ". . . often swayed with the current." Assuming that proprioceptive return is critical to reflex control of ram ventilation in the remora as well, stretch receptor systems still could be activated by passive tail oscillations at frequencies dependent upon water flow velocity.

The author is grateful to Dr. Reuben Lasker for his editorial review of the manuscript, his encouragement, and his sponsorship of that part of the study carried out at the Southwest Fisheries Center, La Jolla, California. Gratitude is also due Directors Dr. Robert L. Edwards (Center Director, Woods Hole, Mass.), and Dr. Brian J. Rothschild (Center Director, La Jolla, Calif.) of the National Marine Fisheries Service for the use of facilities. Special thanks are due Charles L. Wheeler, Aquarium Director (NMFS, Woods Hole, Mass.), his assistant, Harold Ruschky, and Roger Leong, Fisheries Biologist (NMFS, La Jolla, Calif.) for collection and care of the marine fishes used in this study. Data obtained by Sister Mary Arthur Logan during her tenure as a National Science Foundation Summer Research Participant (grant to the University of Massachusetts) also is gratefully acknowledged.

SUMMARY

1. Characteristics of cyclic breathing movements have been examined in a number of fish species at rest and during swimming acceleration to velocities

above those sufficient to induce transfers from active to passive gill ventilation (ram gill ventilation). They were trained to swim in one of several types of swimming tunnels after electrode implants to permit recording of ECG's and ventilatory muscle EMG's.

2. Transfer to the ram mode of gill breathing is marked by a drop-out of individual cyclic breathing movements as swimming increases from rest. When the swimming speed reaches about 65 $cm \cdot sec^{-1}$, most fish that use ram gill ventilation complete conversion to the ram mode (35 to 82 $cm \cdot sec^{-1}$, range of all fish tested). This is equivalent to an across-gill differential pressure of 2.1 cm H_2O as measured with a combined impact-reverse pitot tube as an approximate model. Generally, a fish must be swimming to ram gill ventilate, but some exceptions are noted such as the shark-riding remoras.

3. Control of the transfer between gill ventilation modes appears basically to be a reflex shut-down of rhythmic breathing initiated by mechanoreceptive detection of water flow-velocity and the detection of swimming movements. Reflex transitions between active and passive breathing seem to happen too rapidly for a sufficient change of respiratory gas concentrations to occur and allow triggering of chemoreceptors.

4. No unusual events are detectable in the ECG's of swimming fish as they reach and surpass speeds sufficient to maintain the ram mode. Cardioacceleration varies as between sluggish and active swimmers, but seems to be independent of the mode of gill ventilation used.

5. Generally, demersal fishes that depend mainly upon the opercular phase for active gill ventilation, strongly aided by a well-developed branchiostegal system, do not ram ventilate. Conversely, nearly all species in which the work share of active ventilation is about equally buccal and opercular, probably use ram gill ventilation when they reach the requisite swimming speed. These are mostly midwater to pelagic in habits so for them, transfer of the muscular work of gill ventilation from the branchial to the swimming musculature seems to serve a dual function—a reduction in the cost of breathing, and an improvement in swimming efficiency.

NOTE ADDED IN PROOF

Jones and Schwarzfeld (1974, *Resp. Physiol.*, **21**: 241–254) have revised Jones' earlier estimate (1971) for the oxygen cost of breathing to total metabolism in rainbow trout upward from one to ten per cent on the basis of measurements with hatchery fish.

LITERATURE CITED

ALEXANDER, R. McN., 1970. *Functional Design in Fishes.* Hutchinson, London, 160 pp.

BAILEY, R. M., J. E. FITCH, E. S. HERALD, E. A. LACHNER, C. C. LINDSEY, C. R. ROBINS AND W. B. SCOTT, 1970. *A List of Common and Scientific Names of Fishes from the United States and Canada.* American Fisheries Society, Special Publication No. 6.

BALLINTIJN, C. M., 1972. Efficiency, mechanics and motor control of fish respiration. *Resp. Physiol.*, **14**: 125–141.

BALLINTIJN, C. M., AND G. M. HUGHES, 1965. The muscular basis of the respiratory pumps in the trout. *J. Exp. Biol.*, **43**: 349–362.

Bigelow, H. B., and W. C. Schroeder, 1953. Fishes of the Gulf of Maine. *Fishery Bulletin of the Fish and Wildlife Service,* 53: 1–577.

Brett, J. R., 1964. The respiratory metabolism and swimming performance of young sockeye salmon. *J. Fish. Res. Board Can.,* **21**: 1183–1226.

Brown, C. E., and B. S. Muir, 1970. Analysis of ram ventilation of fish gills with application to skipjack tuna (*Katsuwonus pelamis*). *J. Fish. Res. Board Can.,* **27**: 1637–1652.

Cameron, J. N., and J. J. Cech, Jr., 1970. Notes on the energy cost of gill ventilation in teleosts. *Comp. Biochem. Physiol.,* **34**: 447–455.

Hall, F. G., 1930. The ability of the common mackerel and certain other marine fishes to remove dissolved oxygen from sea water. *Amer. J. Physiol.,* **93**: 417–421.

Heath, A. G., 1973. Ventilatory responses of teleost fish to exercise and thermal stress. *Amer. Zool.,* **13**: 491–503.

Heath, A. G., and G. M. Hughes, 1973. Cardiovascular and respiratory changes during heat stress in rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.,* **59**: 323–338.

Hughes, G. M., 1960a. A comparative study of gill ventilation in marine teleosts. *J. Exp. Biol.,* **37**: 28–45.

Hughes, G. M., 1960b. The mechanism of gill ventilation in the dogfish and skate. *J. Exp. Biol.,* **37**: 11–27.

Hughes, G. M., 1966. The dimensions of fish gills in relation to their function. *J. Exp. Biol.,* **45**: 177–195.

Hughes, G. M., 1973. Respiratory responses to hypoxia in fish. *Amer. Zool.,* **13**: 475–489.

Hughes, G. M., and M. Morgan, 1973. The structure of fish gills in relation to their respiratory function. *Biol. Rev.,* **48**: 419–475.

Hughes, G. M., and J. L. Roberts, 1969. Gill ventilation in the sea-robin, sculpin, scup and toadfish. *Amer. Zool.,* **9**: 1101.

Hughes, G. M., and J. L. Roberts, 1970. A study of the effect of temperature change on the respiratory pumps of the rainbow trout. *J. Exp. Biol.,* **52**: 177–192.

Hughes, G. M., and G. Shelton, 1962. Respiratory mechanisms and their nervous control in fish. Pages 274–364 in O. Lowenstein, Ed., *Advances in Comparative Physiology and Biochemistry.* Academic Press, New York.

Jones, D. R., 1971. Theoretical analysis of factors which may limit the maximum oxygen uptake of fish: The oxygen cost of the cardiac and branchial pumps. *J. Theor. Biol.,* **32**: 341–349.

Labat, R., 1966. *Electrocardiologie chez les poissons Téléostéens: Influence de quelques facteurs écologiques. Ph.D. thesis, University of Toulouse,* 175 pages.

McAllister, D. E., 1968. Evolution of branchiostegals and classification of teleostome fishes. *National Museum of Canada Bull.,* **221**: *Biol. Ser. 77.*

Magnuson, J. J., 1970. Hydrostatic equilibrium of *Euthynnus affinis,* a pelagic teleost without a gas bladder. *Copeia,* **1970** (1): 56–85.

Magnuson, J. J., 1973. Comparative study of adaptations for continuous swimming and hydrostatic equilibrium of scombroid and xiphoid fishes. *Fish. Bull. (U. S.),* **71**: 337–356.

Miller, D. J., and R. N. Lea, 1972. *Guide to the Coastal Marine Fishes of California. California Department of Fish and Game, Fish Bulletin,* **157**: 1–235.

Muir, B. S., and R. M. Buckley, 1967. Gill Ventilation in *Remora remora. Copeia,* **1967** (3): 581–586.

Randall, D. J., 1970a. The circulatory system. Pages 133–172 in W. S. Hoar, and D. J. Randall, Eds., *Fish Physiology, Vol. IV.* Academic Press, New York.

Randall, D. J., 1970b. Gas exchange in fish. Pages 253–292 in W. S. Hoar, and D. J. Randall, Eds., *Fish Physiology, Vol. IV.* Academic Press, New York.

Roberts, J. L., 1964. Metabolic responses of sunfish to photoperiod and temperature. *Helgolaender Wiss. Meeresuntersuch.,* **9**: 459–473.

Roberts, J. L., 1970. Gill ventilation in swimming fish. *Amer. Zool.,* **10**: 516.

Roberts, J. L., 1973. Effects of thermal stress on gill ventilation and heart rate in fishes. Pages 64–86 in W. Chavin, Ed., *Responses of Fish to Environmental Changes.* Charles C Thomas, Springfield.

Roberts, J. L., 1974. Respiratory adaptations of aquatic animals. In press in F. J., and W. B. Vernberg, Eds., *Physiological Adaptation to the Environment.* Intext, New York.

SCHUMANN, D., AND J. PIIPER, 1966. Der Sauerstoffbedarf der Atmung bei Fischen nach Messungen an der narkotisierten Schlei (*Tinca tinca*). *Pflugers Arch. ges. Physiol.*, **288**: 15–26.

SMITH, L. S., J. R. BRETT AND J. C. DAVIS, 1967. Cardiovascular dynamics in swimming adult sockeye salmon. *J. Fish. Res. Board Can.*, **24**: 1775–1790.

SPOOR, W. A., T. W. NEIHEISEL AND R. A. DRUMMOND, 1971. An electrode chamber for recording respiratory and other movements of free-swimming animals. *Trans. Amer. Fish. Soc.*, **100**: 22–28.

SUTTERLIN, A. M., 1967. Effects of exercise on cardiac and ventilation frequency in three species of freshwater teleosts. *Physiol. Zool.*, **42**: 36–52.

SUTTERLIN, A. M., AND R. L. SAUNDERS, 1969. Proprioceptors in the gills of teleosts. *Can. J. Zool.*, **47**: 1209–1212.

VON WAHLERT, G., 1964. Passive Atmung bei Haien. *Naturwissenschaften*, **51**: 297–298.

Reference: *Biol. Bull.,* **148**: 106–116. (February, 1975)

CONTROL OF CORPORA ALLATA FUNCTION IN LARVAE OF *GALLERIA MELLONELLA*

FRANTISEK SEHNAL AND NOELLE A. GRANGER

Institute of Entomology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia and Department of Developmental and Cell Biology, University of California, Irvine, California 92664

In most insects larval development is controlled by juvenile hormone, which is produced by the corpora allata (*CA*). When a threshold concentration of the hormone is reached before a certain time during the larval intermolt period, the next molt gives rise to another larva; but if this concentration is not reached, metamorphosis begins (Novák, 1966). In many species, metamorphosis starts after a determined number of larval instars, but in some species it may occur earlier or later depending on environmental conditions. This indicates that the function of the *CA* is controlled by both internal and external factors, but little is known of the mechanism of this control. In this study we examined the control of the *CA* in the larvae of the waxmoth, *Galleria mellonella* L.

Piepho (1940) showed that it was possible to prevent metamorphosis in *Galleria* by supplying last instar larvae with extra *CA*. The clearest results were obtained when the larva received three complexes of brain-corpora cardiaca-corpora allata (brain-*CC-CA*), but three pairs of *CC-CA* produced similar effects (*CC* cannot be surgically separated from *CA*). Since implantation of three brains into mature larvae had no effect, Piepho ascribed the action of the complexes to *CA* activity.

More recently it has been shown that implantation of brains into freshly ecdysed last instar larvae of *Galleria* induces development of supernumerary larvae (superlarvae) (Sehnal, 1966a; Pipa, 1971). Krishnakumaran (1972) further demonstrated that brains from injured larvae are capable of evoking development of larval-pupal intermediates when implanted into larvae during the second half of the last instar. Pipa (1971) suggested that the implanted brains caused the extra larval molt by stimulating secretion of ecdysone from the host's prothoracic glands, but Krishnakumaran (1972) assumed that the brains stimulate both the *CA* and the prothoracic glands. We have approached this problem experimentally and examined in detail the role of the brain in the control of the *CA*.

Materials and Methods

A laboratory strain of the waxmoth *Galleria mellonella* L. was maintained on an artificial diet at 30° C and 80% r.h. in total darkness (Sehnal, 1966b). Penultimate and last instar larvae of known age were used for the experiments. The following surgical procedures were employed:

Implantations

Most of the implants were parts of the cerebral neuroendocrine complex. The organs were dissected from last instar larvae in insect saline (Novák, 1966) and after 5–40 minutes were implanted into water-anaesthetized host larvae. A V-shaped cut was made with iridectomy scissors in the dorsolateral region of the 5th abdominal segment of the host, and the implants were inserted into the body cavity by means of two fine glass rods.

Allatectomy and denervation of CA

The larva was anaesthetized in water for about 5 minutes, rinsed quickly in 70% ethanol and then in insect saline, and placed dorsal side down in a Petri dish lined with paraffin and filled with insect saline. The neck region of the larva was stretched by means of two pairs of pins which were fixed to the paraffin in the bottom of the dish. The integument of the neck region was cut with a sharp scalpel so as to expose the tips of the tentorium. The muscles near the posterior ends of the tentorium were pulled aside with a hooked needle and the *CC-CA* complex, which was then visible in the incision, was removed with a pair of fine forceps. Since the *CC* and the *CA* are closely attached to each other, it was impossible to remove the *CA* and leave the *CC* intact. The removed glands were either discarded or immediately replaced approximately in their original position. This latter procedure left the insect with denervated glands. Although we handled the glands as gently as possible, some of those inserted back into the body cavity were undoubtedly damaged, so that these insects were effectively allatectomized rather than denervated. In some cases another implant of either brain or *CC-CA* complex was inserted into the neck incision immediately after the removal or replacement of the glands.

Following the operation, the pins fixing the insect were removed, and the insect was blotted on absorbent paper and transferred to a Petri dish. The operated insects were left at room temperature for 24 hours before being supplied with food and returned to 30° C. Some of them received implants three days later, which were inserted into the abdomen in the manner described above.

RESULTS

Inhibition of metamorphosis by various implants

Table I demonstrates that sham operated animals or animals implanted at the beginning of the last instar with three subesophageal ganglia, three pieces of gut, or three sets of thoracic ganglia underwent a normal larval-pupal transformation. The same results were also observed following implantations of different muscles or portions of fat body. The larval-pupal transformation of *Galleria* was suppressed, however, by the implantation of different parts of the cerebral neuroendocrine complex. Implantation of three entire brain-*CC-CA* complexes was the most effective, but all implants containing either brain or *CC-CA* evoked a similar effect. The majority of the insects supplied with these implants molted into morphologically perfect superlarvae (Sehnal and Schneiderman, 1973). Some of them continued to develop as larvae for yet another larval instar (second extra larval instar), and a few of those implanted with three entire complexes underwent as many as four extra larval molts.

TABLE I

Development of larvae supplied with different implants within 24 hours after the last larval-larval ecdysis

Implant†	Number of operated insects	Per cent of insects entering extra larval instars*				
		n + 1 instar	n + 2 instar	n + 3 instar	n + 4 instar	n + 5 instar
Brain	92	61%	17%	0	0	0
CC-CA	59	67%	17%	0	0	0
Brain-*CC-CA* complex	157	89%	74%	24%	9%	2%
Subesophageal ganglion	30	0	0	0	0	0
Thoracic ganglia	21	0	0	0	0	0
Gut	11	0	0	0	0	0
Sham operated	25	0	0	0	0	0

* The remaining insects pupated

† Tissue from three last instar larvae was implanted into each host larva.

Implantations performed at later periods of the last larval instar revealed significant differences in the action of the different parts of the cerebral neuroendocrine complex (Table II). Larvae responded to brains only when they were implanted within 48–60 hours after the last larval-larval ecdysis, but they responded to the implantation of glands as late as 120 hours after the ecdysis. Even when the number of implanted brains was increased to six, larvae older than 60 hours failed to respond. Insects supplied with either three brain-*CC-CA* complexes or with three pairs of *CC-CA* 60 hours or more after the last larval-larval ecdysis molted into various larval-pupal intermediates (Piepho, 1940; Sehnal and Schneiderman, 1973). While the formation of intermediates was common after the implantation of complexes, the implantation of *CC-CA* often led to a great prolongation of the instar, with the insects eventually pupating.

An important characteristic of the action of the implants was their effect on the length of the instar. For example, in experiments with freshly ecdysed last instar larval hosts, the resulting superlarvae ecdysed 108–120 hours after the implantation of brains or complexes, but 168 hours after the implantation of *CC-CA*. The unaffected insects ecdysed as pupae within 192–198 hours after the implantation of brains or complexes, but within 324 hours after the implantation of *CC-CA*. A few of the larvae implanted with *CC-CA* lived for more than two weeks and grew distinctly larger than normal last instar larvae before eventually pupating. This effect was never observed following brain implantations.

Effects of implantations into allatectomized larvae

The implanted glands could prevent metamorphosis by producing juvenile hormone. The brains, however, might act by eliciting precocious secretion of ecdysone from the prothoracic glands, as suggested by Pipa (1971). The high titer of ecdysone would then induce an accelerated molt before any metamorphic change could occur. On the other hand, the implanted brains could also stimulate the host *CA* to secrete juvenile hormone; the increased hormone titer would then

TABLE II

Development of larvae supplied at different times after the last larval-larval ecdysis with implants from 48–120 hr last instar larvae.

Hosts		Development to superlarvae		Development to intermediates		Development to pupae†	
Age (hrs)	Number	Per cent of total insects	Length of instar (hrs)*	Per cent of total insects	Length of instar (hrs)*	Per cent of total insects	Length of instar (hrs)*
Implantation of 3 brains							
12	89	54	120	0	—	45	192
48	9	44	151	0	—	56	180
60	11	9	192	0	—	91	204
72	25	0	—	0	—	100	192
120	33	0	—	0	—	100	198
Implantation of 3 pairs of glands (c. cardiaca-c. allata)							
12	73	74	168	0	—	17	324
48	23	70	159	0	—	27	281
60	20	35	163	10	204	40	254
120	16	6	240	6	264	63	254
Implantation of 3 complexes (brain-c. cardiaca-c. allata)							
12	36	89	108	0	—	11	198
60	64	48	173	24	170	17	214
120	40	0	—	70	238	30	204
Sham operated							
12	25	0	0	0	0	100	197
48	23	0	0	0	0	100	181
72	25	0	0	0	0	100	178
120	25	0	0	0	0	100	192

* The normal length of the last larval instar is 180–190 hours. The age of the host larvae at the time of implantation was ±8 hours.

† Percent of insects which died during the experiments may be calculated from the data listed in the table.

suppress metamorphosis. To distinguish between these two possibilities, brains were implanted into allactectomized larvae.

As shown in Table III, larvae allatectomized in the penultimate instar underwent a precocious metamorphosis. Precocious metamorphosis never occurred in insects from which one corpus cardiacum-corpus allatum complex was removed (half-allatectomy). However, in the last larval instar, neither complete allatectomy nor half-allatectomy had any significant effect on development. Allatectomy also did not alter the response of the larvae to the implantation of *CC-CA*. A majority of the allatectomized insects supplied with glands underwent an extra larval molt,

TABLE III

Development of allatectomized larvae supplied with different implants from 48–120 hr last instar larvae

Operation	Number of larvae*	Development to larvae and intermediates		Development to pupae		Dead‡
		% of survivors	Length of instar† (hr ± SD)	% of survivors	Length of instar† (hr ± SD)	
Host larvae allatectomized 24 hrs after the penultimate larval-larval ecdysis						
Allatectomy	12	0	—	100	not recorded	0
Half-allatectomy	12	100	not recorded	0	—	0
Host larvae allatectomized 12 hrs after the last larval-larval ecdysis						
Allatectomy	7	0	—	100	204 ± 24	2
Allatectomy plus implantation of 3 brains	42	0	—	100	214 ± 21	15
Allatectomy plus implantation of 6 brains	12	0	—	100	245 ± 21	3
Allatectomy plus implantation of 3 pr of glands	36	72	214 ± 48	28	432±201	18
Allatectomy plus implantation of 6 pr of glands	8	100	218 ± 80	0	—	2
Half-allatectomy plus implantation of 3 brains	25	39	142 ± 24	61	257 ± 37	2
Sham operated	21	0	—	100	284 ± 46	3
Host larvae allatectomized 72 hrs after the last larval-larval ecdysis						
Allatectomy plus implantation of 3 brains	10	0	—	100	250 ± 46	3
Allatectomy plus implantation of 3 pr of glands	9	50	415 ± 97	50	569±327	3
Sham operated	35	0	—	100	264 ± 77	6

* Only animals which survived more than 24 hrs after operation are included.

† The first 24 hours after the operation were not included in the instar length.

‡ This column includes animals which either died or did not moult during the three weeks of the experiments.

and the remainder pupated after a delay averaging 18 days. The delay was greater than that observed after the implantations of *CC-CA* into intact larvae and was particularly common in insects allatectomized 72 hours after the last larval-larval ecdysis. Some allatectomized larvae lived for 3–6 weeks after the implantation of *CC-CA* before eventually dying.

The allatectomized insects failed to respond to the implantation of brains, however, and molted into normal pupae. Even the implantation of six brains did not elicit extra larval development. In contrast, three brains were adequate

TABLE IV

Development of larvae with nerves innervating the corpora cardiaca-corpora allata severed and with implants supplied 0 or 72 hrs later

Operation	Number of larvae*	Development to larvae and intermediates		Development to pupae		Dead‡
		% of survivors	Length of instar† (hr ± SD)	% of survivors	Length of instar† (hr ± SD)	
Nerves servered 24 hrs after the penultimate larval-larval ecdysis						
Nerves severed	21	95	not recorded	5	not recorded	
Nerves severed 12 hrs after the last larval-larval ecdysis						
Nerves severed	45	7	156 ± 0	93	328 ± 120	12
Nerves severed and 3 brains implanted	39	48	189 ± 96	52	278 ± 61	12
Nerves severed and 3 brains implanted 72 hr later	30	15	187 ± 36	85	288 ± 67	3
Sham operated	21	0	—	100	284 ± 46	3
Sham operated and 3 brains implanted	8	57	175 ± 12	43	252 ± 42	1
Nerves severed 72 hrs after the last larval-larval ecdysis						
Nerves severed	36	7	360 ± 101	93	384 ± 185	5
Nerves severed and 3 brains implanted	36	20	391 ± 132	80	317 ± 98	1
Sham operated	35	0	—	100	264 ± 77	6
Sham operated and 3 brains implanted	6	0	—	100	308 ± 55	0
Nerves severed 120 hrs after the last larval-larval ecdysis						
Nerves severed	37	3	264 ± 0	95	449 ± 94	5
Nerves severed and 3 brains implanted 72 hr later	11	12	576 ± 0	88	357 ± 62	2
Sham operated	16	0	—	100	271 ± 14	1

* Only animals which survived for more than 24 hrs after the operation are included.

† The first 24 hrs after the operation were not counted.

‡ This column includes animals which either died or did not moult during the four weeks of the experiments.

to induce development of superlarvae in nearly 40% of half-allatectomized insects; the rest of the half-allatectomized larvae pupated with a slight prolongation in the length of the instar. These results clearly demonstrate that the presence of at least one corpus cardiacum-corpus allatum complex is essential for the effect of the implanted brains.

Development of larvae with denervated glands

Table IV demonstrates that freeing the host *CC-CA* of all nervous connections had no effect on the development of penultimate instar larvae. With the exception of a single larva which pupated (probably due to the damage of its *CC-CA* during the operation), all operated insects ecdysed into normal last instar larvae.

Severing the nerves to the *CC-CA* during the last larval instar had no effect on the larval-pupal transformation of 93–95% of the operated larvae, although metamorphosis occurred significantly later than in intact or allatectomized larvae (*cf.* Tables II and III). Suppression of metamorphosis did occur in a few cases. Those affected animals which had been operated 12 hours after the last larval-larval ecdysis produced perfect superlarvae, while those operated at 72 and 120 hours after the ecdysis molted, after about two weeks, into imperfect superlarvae and larval-pupal intermediates. In addition, nearly 10% of all operated insects failed to pupate for as long as four weeks after the operation.

Severing their nervous connections did not prevent the glands of freshly ecdysed last instar larvae (12 hours after the ecdysis) from responding to the implanted brains (Table IV). After the implantation of three brains, about half of these larvae developed into superlarvae; the occurrence of extra larval instars in these animals was thus only slightly less frequent than in intact larvae following the implantation of three brains (Table I). However, when the implantation of brains was postponed until 72 hours after the denervation of the glands, only 15% of the experimental larvae underwent an extra larval molt.

Most significantly, denervating the glands 72–120 hours after the last larval-larval ecdysis restored their sensitivity to the implanted brains. Although intact larvae of this age never respond to implanted brains, 10–20% of the denervated insects molted, after brain implantation, into imperfect superlarvae and larval-pupal intermediates.

Thus severing the nerves to the *CC-CA* complex during the last larval instar partially restores gland activity and also their sensitivity to implanted brains. Following denervation the glands secrete enough juvenile hormone to cause a significant prolongation of the instar and, in a few cases, the development of superlarvae. The low numbers of operated insects undergoing the extra larval development indicate that some inhibition of *CA* function persists after nerve severence. The source of the inhibition could be the brain. However, its inhibitory action appears to be conditioned by the brain's integrity with the rest of the nervous system. When the brain with attached *CC-CA* is transplanted into another larva, the glands resume hormone production and induce extra larval development in the host (*cf.*, Table II). In addition, it is clear that the role of the nervous connections from the subesophageal ganglion to the *CC-CA* complex in the inhibition of gland function has not been defined.

Discussion

Freshly ecdysed last instar larvae of *Galleria mellonella* respond to the implantation of three brains with an extra larval molt. Since this effect also occurs in larvae from which one *CA* has been removed, but not in larvae from which both *CA* have been removed, the implanted brains must exert their effect by activating the *CA* of the host. The activation is not mediated by the host nervous system because brains are also active in larvae in which all nervous connections to the *CC-CA* are severed. In another paper we consider the nature of this blood-borne allatotropic factor (Granger and Sehnal, 1974).

A humoral, presumably neurohormonal, control of the *CA* by the brain has also been demonstrated in various adult insects (*cf.* Cassier, 1967; Engelmann, 1970) and also in some larvae. Dreschner (1960) proposed an allatotropic function for the brain in nymphs of *Periplaneta americana.* Girardie (1965, 1967) showed that neurosecretory cells in the brains of *Locusta migratoria cinerascens* nymphs produce both stimulatory and inhibitory factors affecting the *CA*, although the inhibitory factor may inactivate the released juvenile hormone rather than repress its production. Ozeki (1962) presented indirect evidence for a humoral control of the *CA* in the earwig *Anisolabis maritima*: inactive *CA* became activated when cultured in penultimate instar larvae for ten days. Similarly, Fukuda (1962) showed that transplanted *CA* in *Bombyx mori* often behave in a manner corresponding to the developmental stage of the host.

The sensitivity of *Galleria CA* to the allatotropic factor is maintained for the first 48–60 hours of the last larval instar, after which it seems to be lost. However, the insensitivity can be partly abolished by severing the nerves innervating the *CC-CA* complexes. In a few instances denervation itself, without brain implantation, activates the glands and suppresses metamorphosis. These results indicate that the secretory function of the *CA* is inhibited via the nerves between 48–60 hours after the last larval-larval ecdysis.

The possibility of nervous control of the *CA* was suggested by Scharrer (1946) and later by other authors, but no conclusive evidence for this control in larvae is available. The published data demonstrate that the integrity of the allatal nerves has a different significance for *CA* activity in different species. For example, in the cockroach *Leucophaea maderae*, severing the *CA* nerves has a strong stimulating influence on the glands, and all operated insects undergo an extra larval molt (Lüscher and Engelmann, 1960). In contrast, this operation stimulates extra larval development in only 7% of the cases in *Galleria* (present results) and never in *Bombyx mori* (Bounhiol, 1957). In *Anisolabis maritima*, severing the *CA* nerves causes a partial inhibition of *CA* activity (Ozeki, 1962), an effect opposite to that observed in *Leucophaea maderae*.

These and other data in the literature indicate that insect *CA* are controlled by both activating and inhibiting stimuli which reach the glands either via the haemolymph or via the nerves. The significance of these two mechanisms of transmission appears to be different in different species. In certain stages of development, the effect of one type of stimulus seems to persist, and the glands do not respond to the opposite stimulus. For example, *CA* dissected from *Anisolabis maritima* during the first three quarters of the last larval instar (Ozeki, 1965), or from *Bombyx mori* during the second half of the last larval instar (Fukuda, 1944), remain inactive when transplanted into allatectomized larvae of the penultimate instar; however, the internal milieu of penultimate instar larvae of either species normally has a stimulating influence on *CA* activity (Fukuda, 1962; Ozeki, 1962). Oshiki and Morohoshi (1973) have found that when a certain strain of *Bombyx mori* is exposed to temperature and moisture shocks after the third larval ecdysis, the *CA* become inactive during the fourth larval instar instead of during the fifth, and a premature metamorphosis ensues. When inactive glands from shocked fourth (last) instar larvae are transplanted into allatectomized and untreated fourth instar larvae, the transplanted *CA* become activated and the host larvae undergo a normal fifth larval instar. It was further observed that activation of the *CA* occurs much less frequently when whole brain-*CC*-*CA* complexes are transplanted. In this case the nervous connections to the brain are obviously important for the maintenance of *CA* inhibition.

In summary, we conclude that the activity of the *CA* is determined by an interplay of activating and inhibiting stimuli of varying intensity, whose effects may persist for various lengths of time. Similar mechanisms seem to control the function of the *CA* in adult insects, as was recently demonstrated by Baehr (1973) in *Rhodnius prolixus*.

Studies on volume changes of the *CA* led Kaiser (1949) and Novák (1954) to conclude that the amount of juvenile hormone secreted during larval development depends primarily on the size of the glands. The authors assumed that the concentration of juvenile hormone becomes gradually smaller in succeeding larval instars because the *CA* grow more slowly than the rest of the body. As the result of a disproportion between *CA* volume and body size in a certain instar, the glands do not produce a sufficient concentration of juvenile hormone, and metamorphosis begins. The theory may be valid in considering the phylogenetic origin of insect metamorphosis, but does not seem to apply to certain present-day species. It was disproven in the case of *Leucophaea maderae* (Luscher and Engelmann, 1960) and it is also inconsistent with our data on *Galleria*. In our experiments, removal of one of the two *CA* from penultimate instar larvae undoubtedly caused a great disproportion in the ratio of gland volume to body size, but had no effect on the further development of the larvae. This indicates that more complex mechanisms than the allometric growth of the *CA* control the titer of juvenile hormone in the larva; the nature of some of these mechanisms was previously discussed.

Up to 48 hours after the last larval-larval ecdysis, a larva of *Galleria* may develop either into a perfect superlarva or into a normal pupa, depending on the circulating titer of juvenile hormone (Piepho, 1940; Sehnal and Schneiderman, 1973). Both internal and external stimuli determine which of these developmental possibilities will be realized. Although metamorphosis normally begins in the seventh larval instar, it may occur earlier or later if the insects develop in unfavorable conditions (Sehnal, 1966b).

We assume that after each larval-larval ecdysis, the brain, in response to different internal and external stimuli, is programmed either for larval development or for metamorphosis. When it is programmed for larval development, it releases the allatotropic factor and the *CA* begins secreting juvenile hormone in amounts sufficient to induce larval development. Secretion of the brain hormone, which stimulates the prothoracic glands, seems to be accelerated. On the other hand, when the brain is programmed for metamorphosis, the *CA* are inactivated via their nervous connections between 48 and 60 hours after the ecdysis. The glands *in situ* cannot thereafter be fully activated by the insect's own brain, at least until the end of the last larval instar. This persistent inactivation of the *CA* prevents lethal deviations from normal development, since if the glands resumed their full activity in the course of the instar, inviable larval-pupal intermediates would be produced. Hence, a double-control mechanism—lack of the allatotropic factor and nervous inhibition of the *CA*—assures that the insect develops either into a perfect larva or into a perfect pupa.

The double-control mechanism may play yet another role, namely in governing the precise level of juvenile hormone secretion. Denervation of the *CC-CA*, as well as the implantation of the *CC-CA* into allatectomized larvae, often leads to a significant delay in the larval-pupal ecdysis, similar to that which occurs after administration of low doses of juvenile hormone and its analogues. This prolongation of the instar resembles the diapause which occurs in *Galleria* under certain environmental conditions (Sehnal, 1966b). A similar larval diapause in *Chilo supressalis* (Fukaya, 1962) and a profound larval diapause in *Diatraea grandiosella* (Yin and Chippendale, 1973) are caused by a low level of the juvenile hormone secretion. We assume that the same is true in *Galleria*. Since prolongation of the instar occurs after severance of the nerves to the *CC-CA* complex, the low level of hormone secretion by the *CA* may occur as a result of the partial removal of their nervous inhibition. In another paper we examine variations in the allatotropic activity of the brain during the penultimate and last larval instars of *Galleria* (Granger and Sehnal, 1974). The experiments of Krishnakumaran (1972) indicate that the allatotropic activity of the brain of *Galleria* may also vary in response to a stimulus such as injury. Insects may use this allatotropic control by their nervous system to alter and adjust the secretory activity of the *CA* during their entire development.

One of us (N.A.G.) wishes to thank the National Academy of Sciences U. S. A. for financial support during her stay at the Entomological Institute, CSAV, in Prague. Critical reading of the manuscript by Drs. H. A. Schneiderman, P. J. Bryant, and V J. A. Novák is gratefully acknowledged.

SUMMARY

1. The implantation of three brains into freshly ecdysed last instar larvae which possess at least one of the two corpora allata induces extra larval development. Implanted brains appear to produce a neurohumoral allatotropic factor.

2. Corpora allata seem to be inhibited via their nervous connections 48–60 hours after the last larval-larval ecdysis and become insensitive to the allatotropic factor. Severance of the nerves innervating the corpora allata and adjacent corpora cardiaca induces extra larval development in 7% of the experimental insects and restores sensitivity to the implanted brains in as many as 20%.

3. Severance of the nerves to the corpora cardiaca—corpora allata complexes or implantation of corpora cardiaca—corpora allata complexes into allatectomized larvae in some instances causes a considerable prolongation of the last larval instar. It is suggested that disconnection of the corpora allata from the brain partly removes their inhibition and induces secretion of a low titer of juvenile hormone which is then responsible for the delay in pupation.

LITERATURE CITED

Baehr, J. C., 1973. Contrôle neuroendocrine du fonctionnement du corpus allatum chez *Rhodnius prolixus*. *J. Insect. Physiol.*, **19**: 1041–1055.

Bounhiol, J. J., 1957. La métamorphose se produit, chez *Bombyx mori*, après supression, au dernier stade larvaire, des relations nerveuse entre cérébroïdes et corps allates, cieux-ci restant longtemps imprégnés de neurosécretion. *C. R. Acad. Sci.*, **245**: 1087–1089.

Cassier, P., 1967. La reproduction des insectes et la régulation de l'activité des corps allates. *Ann. Biol.*, **6**: 595–670.

Dreschner, W., 1960. Regenrationsversuche am Gehirn von *Periplaneta americana* unter Berücksichtigung von Verhaltensänderungen und Neurosekretion. *Z. Morphol Ökl. Tiere*, **48**: 576–649.

Engelmann, F., 1970. *The Physiology of Insect Reproduction.* Pergamon Press, Oxford, 307 pp.

Fukaya, M., 1962. The inhibitory action of farnesol on the development of the rice stem borer in post-diapause. *Jap. J. Appl. Ent. Zool.*, **6**: 298.

Fukuda, S., 1944. The hormonal mechanism of larval molting and metamorphosis in the silkworm. *J. Fac. Sci. Tokyo Imp. Univ., Sec. IV*, **6**: 477–532.

Fukuda, S., 1962. Secretion of juvenile hormone by the corpora allata in pupae and moths of the silkworm, *Bombyx mori. Annot. Zool. Japan.*, **35**: 199–212.

Girardie, A., 1965. Contribution à l'étude du controle de l'activité des corpora allata par la pars intercerebralis chez *Locusta migratoria* (L.). *C. R. Acad. Sci.*, **261**: 4876–4878.

Girardie, A., 1967. Controle neurohormonal de la metamorphose et de la pigmentation chez *Locusta migratoria cinerascens. Bull. Biol.*, **101**: 79–114.

Granger, N. A., and F. Sehnal, 1974. Regulation of larval corpora allata in *Galleria mellonella. Nature*, **251**: 415–417.

Kaiser, P., 1949. Histologische Untersuchungen über die Corpora allata und Prothoraxdrüsen der Lepidoptera in Bezug auf ihre Funktion. *Arch. Entwicklungs.-Mech. Organism.*, **144**: 99–131.

Krishnakumaran, A., 1972. Injury induced molting in *Galleria mellonella* larvae. *Biol. Bull.*, **142**: 281–292.

Lüscher, M., and F. Engelmann, 1960. Histologische und experimentelle Untersuchungen über die Auslösung der Metamorphose bei *Leucophaea maderae* (Orthoptera). *J. Insect Physiol.*, **5**: 240–258.

Novák, V. J. A., 1954. The growth of the corpora allata during the post-embryonal development in insects. *Acta Soc. Zool. Csl.*, **18**: 98–133.

Novák, V. J. A., 1966. *Insect Hormones.* Methuen Co., Ltd., London, 478 pp.

Oshiki, T., and S. Morohoshi, 1973. The control of growth and development in *Bombyx mori.* XX. Neurosecretion of the brain-corpora allata system in the trimolters derived from tetramolting silkworms by temperature and moisture shocks. *Proc. Jap. Acad.*, **49**: 353–357.

Ozeki, K., 1962. Studies on the secretion of the juvenile hormone in the earwig, *Anisolabis maritima. Sci. Pap. Coll. Gen. Educ., Univ. Tokyo*, **12**: 65–72.

Ozeki, K., 1965. Studies on the function of the corpus allatum during the last nymphal stage in the earwig, *Anisolabis maritima. Sci. Pap. Coll. Gen. Educ., Univ. Tokyo,* **15**: 149–156.

Piepho, H., 1940. Über die Hemmung der Verpuppung durch Corpora allata. Untersuchungen an der Wachsmotte *Galleria mellonella* L. *Biol. Zbl.*, **60**: 367–393.

Pipa, R. L., 1971. Neuroendocrine involvement in the delayed pupation of space-deprived *Galleria mellonella* (Lepidoptera). *J. Insect. Physiol.*, **17**: 2441–2450.

Scharrer, B., 1946. Section of the nervi corpori cardiaci in *Leucophaea maderae* (Orthoptera). *Anat. Rec.*, **16**: 577.

Sehnal, F., 1966a. Action of the juvenile hormone in the larvae of the waxmoth *Galleria mellonella. Ph.D. thesis, University of Brno*, Brno, Czechoslovakia, 183 pp.

Sehnal, F., 1966b. Kritisches Studium der Bionomie und Biometrik der in verschiedenen Lebensbedingungen gezüchteten Wachsmotte, *Galleria mellonella L.* (Lepidoptera). *Z. Wiss. Zool.*, **174**: 53–82.

Sehnal, F., and H. A. Schneiderman, 1973. Action of the corpora allata and of juvenilizing substances on the larval-pupal transformation of *Galleria mellonella* L. (Lepidoptera). *Acta Ent. Bohemoslov.*, **70**: 289–302.

Yin, C.-M., and G. M. Chippendale, 1973. Juvenile hormone regulation of the larval diapause in the southwestern corn borer, *Diatraea grandiosella. J. Insect Physiol.*, **19**: 2403–2420.

Reference: *Biol. Bull.*, **148**: 117–140. (February, 1975)

UPTAKE AND UTILIZATION OF DISSOLVED GLYCINE BY *AURELIA AURITA* SCYPHISTOMAE: TEMPERATURE EFFECTS ON THE UPTAKE PROCESS; NUTRITIONAL ROLE OF DISSOLVED AMINO ACIDS

J. MALCOLM SHICK[1]

Department of Biology, Texas A & M University, College Station, Texas

During the last decade there has been a considerable renewal of interest in the uptake of dissolved organic matter (especially amino acids) by marine invertebrates. Among the goals of these investigations is the elucidation of the importance of dissolved free amino acids to the nutrition of these animals (for recent reviews and bibliographies, see: Johannes, Coward and Webb, 1969; Stephens, 1972; Dixit, 1973; Schlichter, 1973). Most experiments attempting to demonstrate a nutritive role of these substances have utilized well fed animals presumably having high levels of metabolic substrates, although *a priori*, it is in the starved animal that one might expect a significant supplemental nutritional contribution by dissolved compounds.

There appears to be very little direct evidence supporting the hypothesis that dissolved amino acids (at environmentally realistic concentrations) are in fact an energy source for marine invertebrates. Stephens (1967) has pointed out that not all of the assimilated material is necessarily oxidized, but that it may also exert a sparing effect on a variety of metabolic pathways and on growth and reproductive processes. This implies that there need not necessarily be a net uptake of these compounds for them to be nutritionally significant. In the absence of data regarding the total flux of amino acids, an alternative course of investigation is to make qualitative and quantitative comparisons of biochemical, physiological and developmental processes among fed, starved and starved/amino acid-exposed animals. It is this approach that has been taken in the present study of *Aurelia aurita* polyps (scyphistomae) using dissolved glycine.

The uptake of dissolved free amino acids generally follows Michaelis-Menten kinetics, and while this does not necessarily imply that the uptake process is enzyme-mediated, the calculation of the kinetic constant K_t permits comparisons of the affinities of the amino acid uptake systems among different groups of animals. To date, the K_t values reported for marine invertebrates have been discussed almost exclusively in terms of the animals' adaptations to the concentrations of dissolved free amino acids in their respective habitats (Southward and Southward, 1972a, 1972b; Stephens, 1972), although an additional consideration has been demonstrated by Dixit (1973), who found ontogenetic differences in K_t for glycine uptake by a sea urchin. In view of the known influence of temperature on enzyme kinetics in poikilotherms (Hochachka and Somero, 1973), the investigation of the possibility of an analogous effect of temperature on the kinetics of amino acid uptake is clearly indicated.

---

[1] Present address: Department of Zoology, University of Maine, Orono, Maine 04473.

Somewhat surprisingly, temperature effects on uptake *per se* of dissolved organics by marine invertebrates remain generally unexamined, although Stephens (1962a) presented Q_{10} values for amino acid uptake by the maldanid polychaete *Clymenella torquata* and for glucose uptake by the coral *Fungia scutaria* (Stephens, 1962b). Likewise, whether or not the uptake process is subject to temperature acclimation is unknown. Considering the documentation of such effects on oxygen consumption and other parameters in marine invertebrates, it becomes obvious that a complete understanding of the significance of the uptake and utilization of dissolved amino acids must include an awareness of the thermal sensitivity of these processes.

MATERIALS AND METHODS

Experimental animals

A clonal culture of *Aurelia aurita* scyphistomae from Corpus Christi, Texas was begun with a single animal from the culture isolated by Spangenberg (1964). The stock culture was maintained at room temperature (21°–24° C) in iodine-free, 30‰ artificial seawater to prevent strobilation. Polyps were fed *Artemia salina* nauplii twice weekly and food residues were removed from the culture bowls 1–3 days after each feeding. The water was changed monthly. A somewhat larger, genetically heterogeneous culture was maintained under conditions identical to those above, and polyps from this culture were used only in the determination of internal free amino acid pools.

Polyps of *A. aurita* from the York River, Virginia were provided by R. E. L. Black, College of William and Mary. These animals have a lower optimum salinity for growth and asexual reproduction than do the Texas scyphistomae, and were therefore maintained at 25‰. All other details of culture maintenance were as described above. These polyps were used only in the temperature acclimation studies described below, and to distinguish them from the Texas animals they are always specifically referred to as "Virginia" polyps or scyphistomae.

Temperature and starvation effects on glycine uptake

Texas polyps from the clonal culture were fed, brought to one of the acclimation temperatures (12°, 15°, 20°, 25°, 30° and 35° C) over periods ranging from 3–9 days, and again fed once the desired temperature had been attained. One group of polyps at each temperature was then fed twice weekly for 14 days, the last feeding being 2 days before the acclimated glycine uptake experiments were performed. A second group was deprived of food throughout the 14-day period.

At the end of the acclimation period, groups of 10 polyps of uniform size were removed from the culture dishes, adhering debris was removed with a pipet, and the animals were washed 4 times with Millipore filtered (0.45 μ pore size) artificial seawater at the acclimation temperature and salinity. Preliminary experiments in which polyps were preincubated for 21 hr in streptomycin sulfate (200 mg/l seawater) indicated that the above decontamination procedure was sufficient to eliminate any detectable effects of bacteria on total glycine uptake or distribution of radioactivity in the various fractions. Therefore, to minimize external variables, streptomycin was not used in the main body of experiments.

Decontaminated polyps were transferred to acid-rinsed, sterile test tubes in 0.5 ml of artificial seawater (30‰) at the acclimation temperature. To each tube was added 1.0 ml of water at the acclimation temperature and salinity containing [U-^{14}C] glycine (New England Nuclear), so that the final concentration of labeled glycine in the exposure medium was 0.80 μM. This concentration is ecologically realistic (*cf.* Webb and Wood, 1966; Siegel, 1967; Bohling, 1970, 1972; Clark, Jackson and North, 1972).

Preliminary experiments with Texas polyps at 20° C demonstrated that glycine uptake from a medium with an initial concentration of 0.80 μM is linear for at least 120 min; therefore, an exposure period of 1 hr was chosen for all subsequent experiments.

Polyps were exposed to the labeled medium in sealed test tubes for 1 hr at the acclimation temperature, quickly rinsed in 3 changes of artificial seawater and extracted overnight in 1.5 ml of 80% ethanol. The CO_2 was collected throughout the exposure period in 10% KOH on ground glass rods imbedded in the stoppers sealing the individual tubes; after removal of the animals, the exposure medium was acidified with 2 N HCl to drive off any remaining CO_2, which was also trapped in KOH. The extracted polyps were rinsed in 3 changes of clean ethanol and digested overnight in 0.5 ml of NCS Solubilizer (Amersham-Searle). Samples of ethanol extracts, EtOH insoluble material and KOH were prepared for counting as described in Shick (1973), and all samples were counted in Aquasol Universal L. C. S. Scintillator (New England Nuclear). Aliquots of the radioactive media were taken before and after the exposure period, and after acidification in the latter case. All samples were corrected for background and for quenching by use of both internal and external standards. Dry weights for each experimental group of animals were determined as in Shick (1973), with the addition that dried polyps were rinsed of salt before a second drying and reweighing.

The above exposure, CO_2 trapping, extraction, counting and weighing procedures were followed in all other experiments, with modifications described where appropriate. Unless otherwise noted, all experiments were performed in iodine-free artificial seawater of 30‰.

Acclimated rates of glycine uptake by fed (2-day) and starved (14-day) polyps were compared by Student's *t* test and by two-way analysis of variance (*F* test) performed on data obtained at temperatures common to both groups.

Temperature acclimation of glycine uptake rate

Groups of Texas polyps were maintained at 17° C (cold acclimated) and at 27° C (warm acclimated) for 2 weeks. Polyps were fed twice weekly, the last feeding being 2 days prior to the performance of uptake experiments at 17°, 22° and 27° C. An additional group was acclimated to 15° C, with uptake rates being determined at 15°, 22.5° and 30° C.

Groups of Virginia scyphistomae were gradually brought to 30‰ and maintained at 17° and 27° C under the same conditions as the Texas polyps. Other experimental procedures were also identical except that, due to their larger size, polyps were exposed to glycine in groups of 2 uniformly sized individuals at each temperature. Likewise, dry weight determinations on groups of 2 polyps were made as previously described.

Acutely determined rates of glycine uptake (*i.e.*, rates determined at temperatures other than those to which the animals were acclimated) were compared within each population using Student's *t* test and analysis of variance (*F* test).

Kinetics of glycine uptake

Groups of Texas polyps were fed twice weekly and acclimated to temperatures of 12°, 17°, 22°, 27° and 32° C for 14 days, at the end of which time they were exposed in groups of 5 to varying concentrations (0.12–101.36 μM; 3–5 replicates per concentration) of [U-^{14}C] glycine at their respective acclimation temperatures. A group of polyps maintained for 2 weeks at 20° C was placed in artificial seawater containing 1.5 μM potassium iodide and raised to 27° C to induce strobilation. Newly-liberated ephyrae were transferred to iodine-free water at 27° C and used in kinetic determinations at this temperature. The constants V_{max} (maximum uptake rate) and K_t (numerical equivalent of external glycine concentration at half-maximal uptake rate) were calculated from data at each temperature by means of least squares linear regression analyses performed on Eadie-Hofstee plots (v *vs.* v/[S]) of the Michaelis-Menten equation.

$^{14}CO_2$ production

Recently fed (2-day) and starved (14-day) Texas polyps were exposed to 0.80 μM [U-^{14}C] glycine for 1 hr as described above, and the CO_2 was collected throughout the exposure period. Other groups of polyps were thrice rinsed in clean artificial seawater following the exposure period and transferred to unlabeled medium, and CO_2 was collected after exposure plus incubation periods totalling 2, 3, 4, 6 and 8 hr.

In another series of experiments, groups of fed and starved Texas polyps were each exposed for 1 hr to either 1.28 μM [U-^{14}C] glycine or to 1.28 μM [2-^{14}C] glycine. Animals were incubated for 5 hr beyond the exposure period, during which time CO_2 was collected from all experimental groups. Samples were corrected for the specific activities of the two labeled glycine stock solutions in order to determine the relative contributions of the carboxyl and of the alpha carbon to $^{14}CO_2$ production by fed and starved polyps.

Effects of prolonged starvation

Seven groups of 20 clonal Texas scyphistomae were fed *ad libitum* during acclimation to 20° C. All groups were then decontaminated and placed separately in 25 ml of Millipore filtered artificial seawater and subjected to one of the following treatments. Two groups ("starved") were maintained at 20° C without food, the polyps being rinsed and sterile culture dishes and Millipore filtered water being changed daily for 56 days. The third ("starved/alanine-exposed") and fourth ("starved/glycine-exposed") groups were similarly treated, but in addition received 0.10 μM L-alanine or 0.80 μM glycine, respectively, in the daily renewed water; these concentrations approximate those found in estuarine and coastal waters. The fifth group ("starved/glucose-exposed") received 0.27 μM D-glucose, so that while this group had no external nitrogen source, it received approximately the same amount of carbon as did the starved/glycine-exposed polyps. The sixth and seventh groups ("fed") were fed twice weekly during the entire 8-week period.

Buds produced asexually by all groups were counted daily for the duration of the experiment. All buds were removed as soon as they detached from the parent scyphistomae, since increased population density is known to affect the rate of asexual reproduction in *Aurelia* polyps (Coyne, 1973).

Bacterial counts of the water of each group except the fed were made on days 10, 36 and 56. One milliliter of culture water was added to 15 ml of medium containing 1.5 g Bacto-Peptone and 0.5 g Bacto-Agar (Difco Laboratories) per 100 ml of artificial seawater. Appropriate blanks were also prepared, and all plates were incubated at 20° C for 48 hr. While the plate method does not give an absolute count of bacterial cells per unit culture water (Wiebe, 1971), it provides a relative basis for comparisons among experimental cultures.

Following the 56-day maintenance period, polyps were placed in artificial seawater containing 1.5 μM potassium iodide and moved to a 27° C incubator to induce strobilation, and were examined daily for at least 14 days.

Oxygen consumption rates

Several groups of scyphistomae from the clonal culture were fed, brought to 20° C and again fed. One group was then fed twice weekly during maintenance

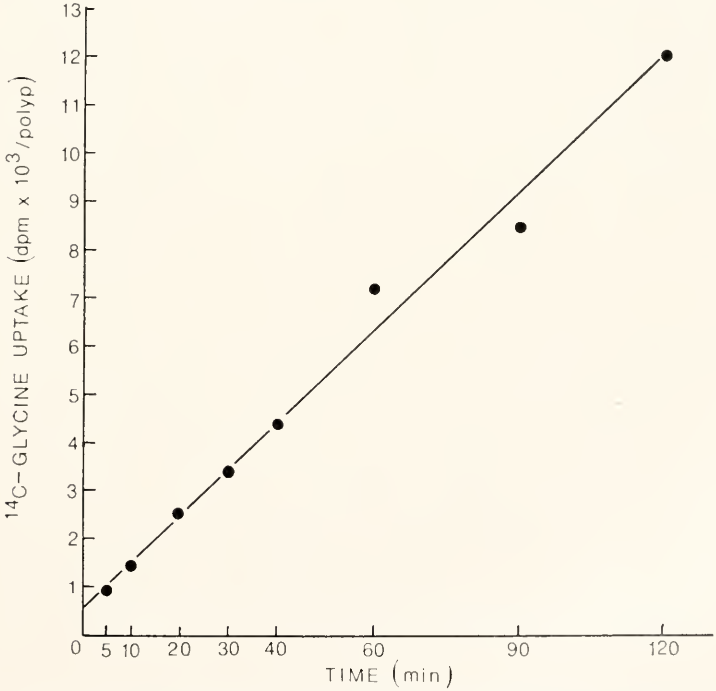


FIGURE 1. Uptake of [U-^{14}C] glycine from an initial concentration of 0.80 μM by groups of 10 Texas polyps as a function of exposure time.

at this temperature for 14 days, while the other 3 groups were deprived of food throughout this period. One group of starved polyps was exposed to 0.80 μM glycine during the last 20 hr of the food deprivation period. Another group of starved animals was exposed to the same concentration of glycine for 1 hr immediately prior to oxygen consumption determinations and a third group of starved polyps remained unexposed to exogenous glycine.

Groups of 50 fed polyps were decontaminated and placed in Millipore filtered artificial seawater at 20° ± 0.1° C in 53-ml vessels equipped with magnetic stirrers. Oxygen depletion in the sealed vessels was monitored continuously with a Yellow Springs Instrument Company Model 5450 polarographic electrode connected to a Model 54 oxygen meter. Following each experimental run, the oxygen consumption by the electrode alone was determined and subtracted from the experimental value. Identical procedures were followed in determinations of oxygen consumption rates in groups of 100–150 starved and starved/glycine-exposed polyps.

Polyps were dried at the completion of their respective runs. All oxygen consumption rates are expressed in terms of dry weight, and were compared using the Student-Newman-Keuls test.

Free amino acid pools

Groups of 100 fed and starved Texas polyps acclimated to 20° C and 30‰ were decontaminated and homogenized in 0.5 ml of absolute ethanol in an ice bath. The homogenate was centrifuged at 20,000 *g* for 20 min, the pellet dried and weighed, an aliquot of the supernatant removed for determination of total ninhydrin positive substances (NPS) according to the method of Clark (1964), and the remainder evaporated to dryness *in vacuo*. The residue was redissolved in 1% HCl and subjected to ion-exchange chromatography on a Beckman 120A amino acid analyzer using a 0.2 N citrate buffer at pH 3.25 and 4.28 on a 55-cm column of PA-28 resin at 55° C. Basic amino acids were not determined.

RESULTS

General

Glycine uptake by Texas polyps is linear for at least 120 min (Fig. 1). An exposure period of 1 hr was therefore chosen for all uptake experiments.

When considering all uptake experiments performed, irrespective of temperature or nutritive state, the range of dry weights of Texas polyps was 0.038–0.080 mg/polyp. In any one experiment, the dry weight of the fed animals never exceeded that of the starved by more than a factor of 2, and a weight correction for glycine uptake rate was not employed. Similarly, the weight range for Virginia polyps was 0.220–0.310 mg/polyp, and no weight correction has been made. Due to the considerable difference in weight between individuals of the 2 populations, however, their weight-specific glycine uptake rates may not be strictly comparable, and no such comparison has been attempted.

When the activities of the exposure media, ethanol soluble and insoluble materials, $^{14}CO_2$, and water and ethanol rinses were monitored, recovery of radioactivity ranged from 89–103% with a mean of 95.7%.

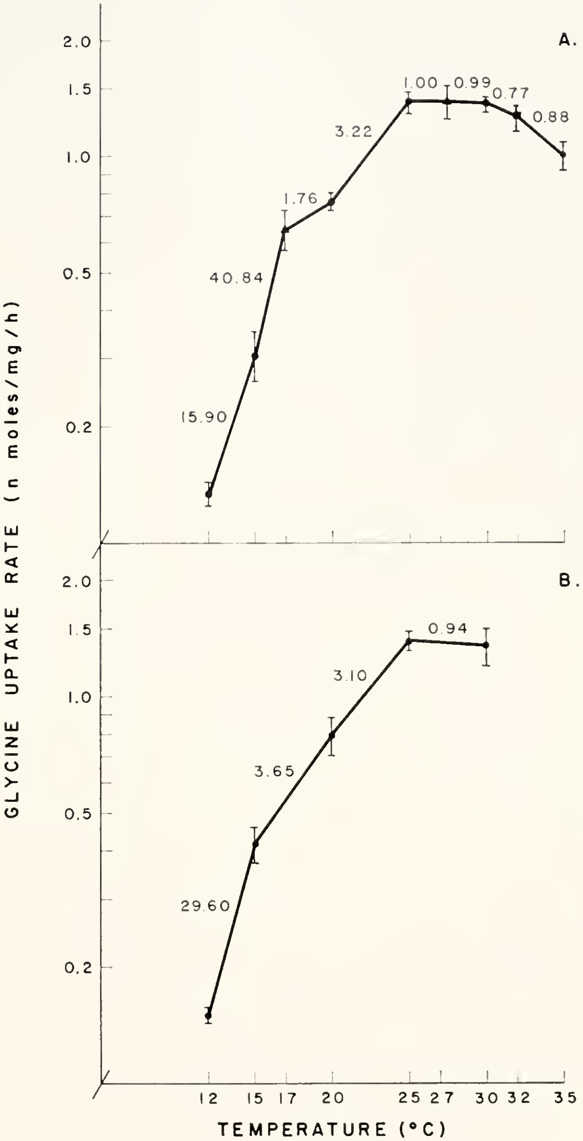


FIGURE 2. Acclimated [U-^{14}C] glycine uptake rates in fed (A) and starved (B) Texas polyps. Each point represents the mean of 5 groups of 10 animals ± SD. Included in (A) are values obtained in kinetic (squares) and temperature acclimation and kinetic (triangles) experiments. Q_{10} values given for each temperature interval.

Temperature and starvation effects on glycine uptake

Fed and starved polyps showed 100% survival at all acclimation temperatures, with the exception of 35° C where the starved animals exhibited 100%

TABLE I

Per cent of total radioactivity present as $^{14}CO_2$ *in recently fed Texas polyps exposed to* $[U\text{-}^{14}C]$ *glycine for 1 hr.* Q_{10} *values for each temperature interval given in parentheses.*

Temperature										
12° C		15° C		20° C		25° C		30° C		35° C
0.02		0.05		0.31		0.48		0.66		0.37
	(21.20)		(38.44)		(2.40)		(1.89)		(0.31)	

mortality. While polyps at 10° C remained contracted, they gave no evidence of cyst formation, and did not survive prolonged exposure to this temperature. Feeding was observed at all acclimation temperatures.

Acclimated rates of glycine uptake for fed and starved polyps are shown in Figure 2. Additional comparable values obtained in kinetic and temperature acclimation experiments are plotted in this figure, although these data were not included in the statistical analyses. The acclimated uptake rate-temperature curve for fed animals indicates extreme thermal sensitivity of uptake in the intervals above the lower lethal limit, reduced sensitivity at intermediate temperatures, thermal insensitivity over the range of midsummer temperatures, and declining uptake as the incipient high lethal level is approached. The curve for starved polyps is virtually identical to that of the fed animals, although the stress of food deprivation did not permit survival at the highest temperature.

Student's *t* tests revealed significant differences in glycine uptake rates between fed and starved polyps only at 12° and 15° C ($P < 0.02$; $P < 0.01$, respectively). Although the gastrovascular cavities of most animals were empty of food residues 2 days after feeding, it is likely that a portion of the 12° C- and 15° C-acclimated polyps' weights was due to unassimilated and metabolically inactive materials, as evidenced by these polyps' longer retention of the color imparted by the carotenoids of the *Artemia* nauplii, leading to a lower apparent weight-specific uptake rate, and possibly accounting for the observed differences. Two-way analysis of variance revealed no significant effect of nutritive state ($P > 0.25$) or of nutritive state-temperature interaction ($P > 0.50$) on acclimated glycine uptake rates, while the effect of temperature was very highly significant ($P < 0.001$).

The per cent of radioactivity present as $^{14}CO_2$ (Table I), which may be taken as an index of metabolic activity, shows a pattern of temperature sensitivity similar to that of glycine uptake. For convenience of comparison, Q_{10} values are given for each temperature interval, but it must be noted that $^{14}CO_2$ production is expressed in terms of relative, not absolute, rates. The considerable increase in the rate of glycine uptake in the 15° to 20° C interval is paralleled by a six-fold increase in relative $^{14}CO_2$ production over this range of temperature. Like glycine uptake, $^{14}CO_2$ production declines above 30° C, a manifestation of the effects of thermal stress.

Temperature acclimation of glycine uptake rate

Acutely determined dissolved glycine uptake rates in cold acclimated (15°, 17° C) and warm acclimated (27° C) Texas scyphistomae, and in similarly acclimated Virginia polyps (omitting the 15° C-acclimated group) are shown in Figure 3. It is immediately apparent that both sets of curves exhibit the comparatively rare pattern of reverse translation (Prosser, 1964), wherein the curve

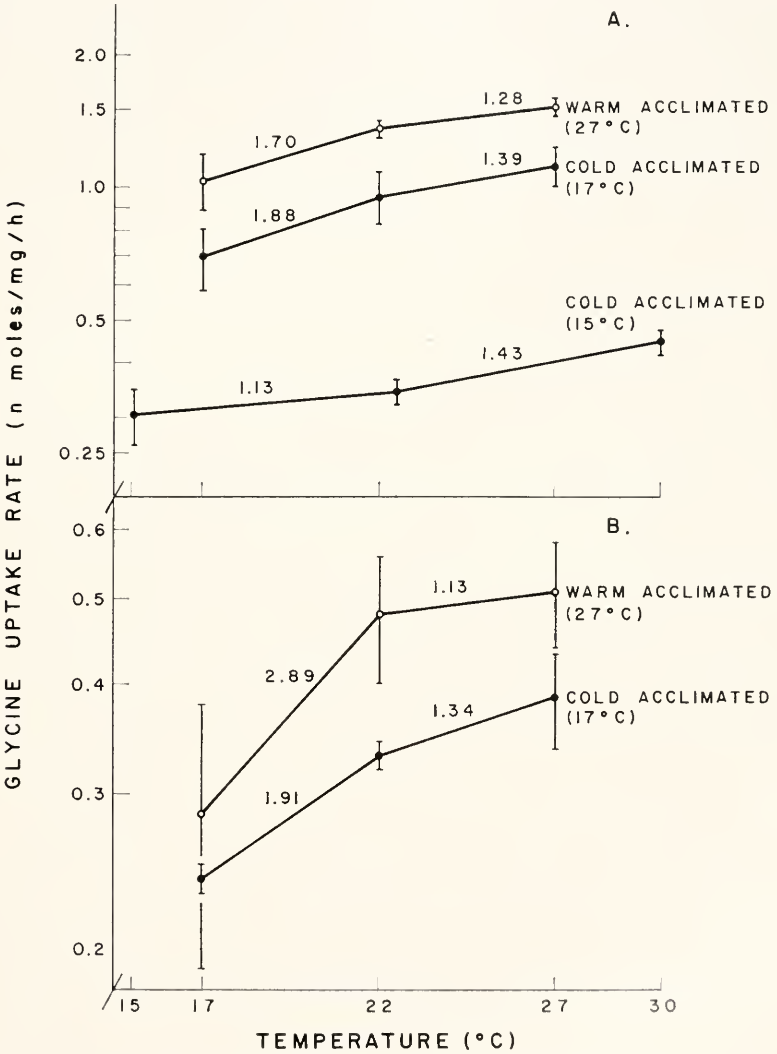


FIGURE 3. Acute [U-^{14}C] glycine uptake rates in Corpus Christi, Texas polyps (A), and in York River, Virginia polyps (B). Each point in (A) represents the mean of 5 groups of 10 animals ± SD; each point in (B) represents the mean of 5 groups of 2 animals ± SD. Q_{10} values given for each temperature interval.

of the warm acclimated animals is displaced upward and to the left of that of the cold acclimated individuals. Student's *t* tests performed on acute rates in 17° C- and 27° C-acclimated animals within both populations revealed significant differences ($P < 0.025$) in all comparisons except the Virginia polyps at 17° C (see Fig. 3B). Divergence between the curves of the 17° C- and 27° C-acclimated polyps in both populations, as determined by analysis of variance, is significant (Texas: $P < 0.005$; Virginia: $P < 0.01$).

The acute measurements were made after the acclimated rates of uptake had been determined. The latter experiments had indicated that 17° C was the threshold below which glycine uptake rates in Texas polyps exhibit extreme thermal sensitivity (Fig. 2A), and accordingly, 17° C was chosen as the cold maintenance temperature. Acute determinations performed on Texas polyps maintained at 15° C, below the aforementioned threshold, did in fact reveal a considerable depression in uptake rates relative to the 17° C-acclimated animals (Fig. 3A). The ecological significance of the 17° C threshold is not obvious.

Kinetics of glycine uptake

The constants K_t and V_{max} at each acclimation temperature are given in Table II. Values of both constants are directly related to acclimation temperature. The correlation coefficients indicate a high degree of linearity in regression analyses of Eadie-Hofstee plots of data at all temperatures, and suggest that the diffusion component of uptake is not large. The latter also follows from the large concentration gradient against which glycine is taken up by the polyps. The possibility of glycine transport in *Aurelia* polyps by carrier-mediated exchange diffusion has not been examined, although Wong (1971) and Stephens (1972) have shown that this is not the case in other marine invertebrates. When all acclimation temperatures and concentrations of labeled glycine in the media are considered, the amount of radioactivity recovered as $^{14}CO_2$ and ethanol insoluble material ranges from 0.7–4.3% of the total uptake; thus, the effects of temperature on K_t and V_{max} are genuine and not artifacts resulting from the metabolic removal of labeled glycine from the soluble pool.

The K_t and V_{max} for glycine uptake by ephyrae at 27° C are also given in Table II. While there is little difference between polyps and ephyrae in the

TABLE II

Effects of temperature on K_t and V_{max} of glycine uptake by Texas polyps. Values determined from linear regression analyses of Eadie-Hofstee plots of data.

Temperature (°C)	K_t ($\times 10^{-5}$ M)	V_{max} (n moles mg dry weight^{-1} h^{-1})	Correlation coefficient
12	0.79	1.61	−0.984
17	0.89	6.49	−0.967
22	1.23	20.16	−0.971
27	2.35	39.32	−0.947
27 (ephyrae)	2.49	93.51	−0.925
32	3.89	64.13	−0.963

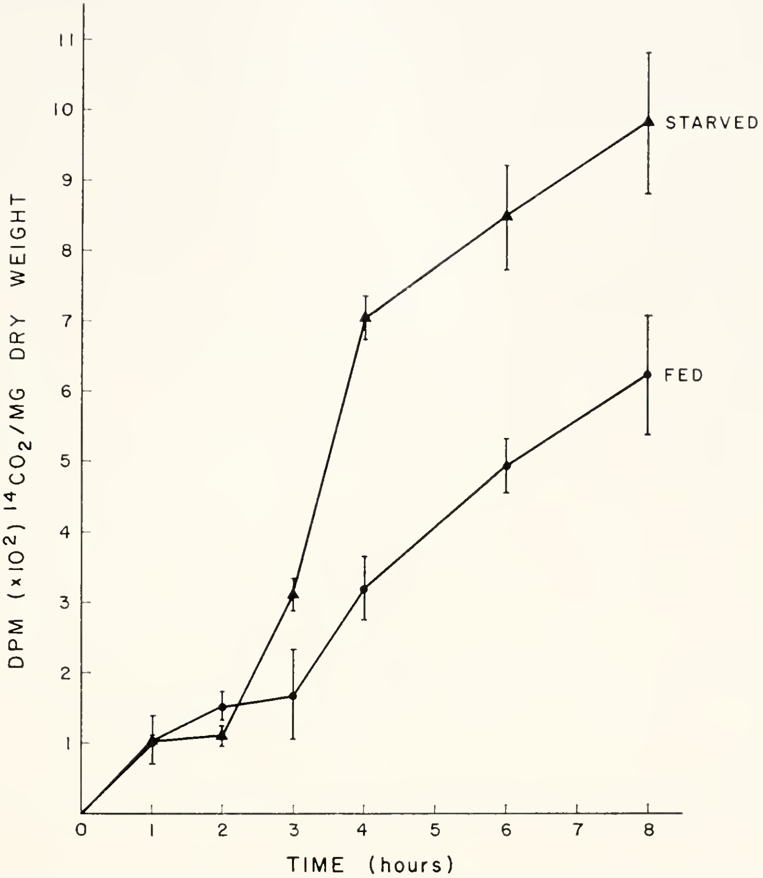


FIGURE 4. Time course of $^{14}CO_2$ production by fed (circles) and starved (triangles) Texas polyps following a 1-hr exposure to [U-^{14}C] glycine. Each point represents the mean of 4 groups of 10 animals ± SD.

values of K_t, ephyrae have a somewhat higher weight-specific V_{max}. The possible significance of these observations is discussed below.

$^{14}CO_2$ production

The time course of $^{14}CO_2$ production by fed and starved polyps exposed to 0.80 μM [U-^{14}C] glycine is shown in Figure 4. There is no apparent difference in the rate of $^{14}CO_2$ production between the 2 groups during the first 2 hr, following which there is a large increase in the rate of its production by the starved polyps.

The total $^{14}CO_2$ production by the starved polyps then stabilizes at a level almost double that of the fed animals, similar to the results of the longer-term incubations reported by Shick (1973). Conversely, the percentage incorporation of labeled glycine into ethanol insoluble materials during the 1-hr exposure period decreases after 2 weeks of starvation (Table III).

TABLE III

Per cent of total radioactivity present as ethanol insoluble material in fed and starved Texas polyps exposed to [U-^{14}C] glycine for 1 hr.

	Temperature					
	12° C	15° C	20° C	25° C	30° C	35° C
Fed	1.40	2.18	2.59	2.57	2.03	3.28
Starved	1.05	0.76	0.69	1.89	1.99	—

It was found that 82% of the $^{14}CO_2$ produced by fed animals was derived from the carboxyl carbon, 18% being derived from the alpha carbon (Table IV). In starved animals these values are 64% and 36%, respectively, indicating a more complete breakdown of the glycine molecules taken up from solution by these polyps. Corrected values for labeled ethanol soluble and insoluble materials are also given in Table IV; chi-square analyses revealed no significant differences between fed and starved polyps in derivation of labeled carbon in these fractions.

Effects of prolonged starvation

Quantitative effects of prolonged starvation on strobilation and budding, with and without concomitant exposure to dissolved organic matter (DOM), are given in Table V, as are bacterial counts of the water from the groups deprived of solid food. Survival during 56 days of starvation was excellent, the figure of 97.5% in the "starved" groups being due to loss of a single damaged polyp during the first week.

Production of buds by the fed polyps continued throughout the entire period, but ceased after 14–15 days in all other groups. Likewise, there is little difference among the latter groups in total number of buds produced.

The minimum time to strobilation initiation (in this study, referring to the first evidence of either constriction or flattening of the polyp) after exposure to

TABLE IV

Uptake of radioactive glycine (Gly) and distribution of label among various fractions corrected for specific activities of the two radioactive glycine stock solutions, and expressed as dpm/mg dry weight ± SD, where n = 3 groups of 10 Texas polyps at both nutritive states and glycine sources.

Nutritive state	Fraction	Radioactive source		Derivation of ^{14}C in fractions (carboxyl-^{14}C; α-^{14}C)
		[U-^{14}C] Gly	[2-^{14}C] Gly	
Fed	CO_2	648 ± 83	115 ± 23	(82%; 18%)
Fed	EtOH soluble	242,034 ± 9816	263,447 ± 18,215	(48%; 52%)
Fed	EtOH insoluble	18,901 ± 2199	19,400 ± 743	(50%; 50%)
Starved	CO_2	4112 ± 366	1465 ± 213	(64%; 36%)
Starved	EtOH soluble	329,675 ± 23,952	350,659 ± 17,240	(49%; 51%)
Starved	EtOH insoluble	17,753 ± 2191	20,250 ± 1188	(47%; 53%)

TABLE V

Results of prolonged starvation (56 days at 20° C), with and without concomitant exposure to dissolved organic compounds, on strobilation and budding in Texas polyps. See text for discussion of qualitative differences among groups.

	Fed*	Starved*	Starved/ alanine	Starved/ glycine	Starved/ glucose
% Survival	100, 100 (100)	95, 100 (97.5)	100	100	100
Total buds produced by 20 polyps	217, 263	17, 21	16	19	17
Time to 1st observed strobilation initiation (days)	3, 3	5, 5	3	3	3
% Strobilating	100, 100 (100)	20, 25 (22.5)	100	100	100
Ephyrae produced per polyp	3.8, 3.5	1.0, 1.0	1.6	1.2	1.4
Abnormal ephyrae/total ephyrae	3/77, 4/69	2/4, 3/5	5/32	3/24	15/27
Bacterial colonies/ml culture water (average of determinations on days 10, 36 and 56)	—	7.4, 6.0	3.4	2.8	4.5

* Data for 2 groups of 20 polyps.

iodide and temperature increase also varied among the treatment groups. The shorter time was observed in the fed and the starved/DOM-exposed groups, and the longer in the starved.

The most dramatic and informative difference among the treatment groups is the per cent of animals strobilating. All polyps in the fed and in the starved/DOM-exposed groups produced ephyrae, while the response was reduced to 22.5% in the starved animals. Among the groups deprived of solid food, ephyra production was exclusively via monodisk strobilation in the starved polyps, while at least some of the starved/DOM-exposed polyps produced 2 ephyrae. Typical polydisk strobilae were observed in the fed groups.

A high percentage of the ephyrae produced by starved and starved/glucose-exposed polyps exhibited severe developmental and morphological anomalies. Such abnormalities were seen less frequently among fed and starved/amino acid-exposed groups. An ephyra was considered to be abnormal if it had other than the normal 8 bifurcated marginal lobes, or if it was grossly misshapen. The significance of these observations is considered below.

Zobell and Feltham (1938) presented evidence of bacteria serving as a food source for a number of marine invertebrates, and DiSalvo (1971) and Sorokin (1973) have extended this observation to corals. Percival (1923) demonstrated that *Aurelia* scyphistomae ingest carmine particles transported to the mouth in ciliated tracts on the body surface. These lines of evidence, taken together, suggest that scyphistomae could feed on bacteria present in the water and such a mechanism, if employed by the polyps, could overshadow the nutritive role of dissolved organic compounds suggested by the results of the above experiments. However, the daily changing of the culture dishes and the Millipore filtered water, the daily rinsing of the polyps, and the 20° C maintenance temperature kept bacterial growth to comparable minima in the starved cultures (Table V). It does not seem likely that the minimal growth affected the experimental results.

Oxygen consumption rates

Oxygen consumption rates in fed, starved and starved/glycine-exposed polyps are given in Figure 5. The rates are mean values for oxygen consumption rates over a 5-hr period by 4 groups of polyps at each experimental condition. Attempts to correlate oxygen consumption with increases in $^{14}CO_2$ production in the 2–4 hr interval after glycine exposure (Fig. 4) proved inconclusive. While starved polyps exposed to glycine for 1 hr exhibited no consistent increase in oxygen consumption in the 2–4 hr interval during the continuous measurements, the rates were highly variable both among groups and within a given group over the time course of the determinations, perhaps reflecting metabolic instability during the activation of catabolic pathways.

The rates in the 3 groups deprived of solid food are significantly lower than the rate in fed polyps ($P < 0.001$ in all cases). The rate in starved/glycine-exposed (20 hr) polyps is significantly greater ($P < 0.05$) than that in the starved scyphistomae. The rate in starved/glycine-exposed (1 hr) polyps does not differ significantly ($P > 0.05$) from that in starved polyps.

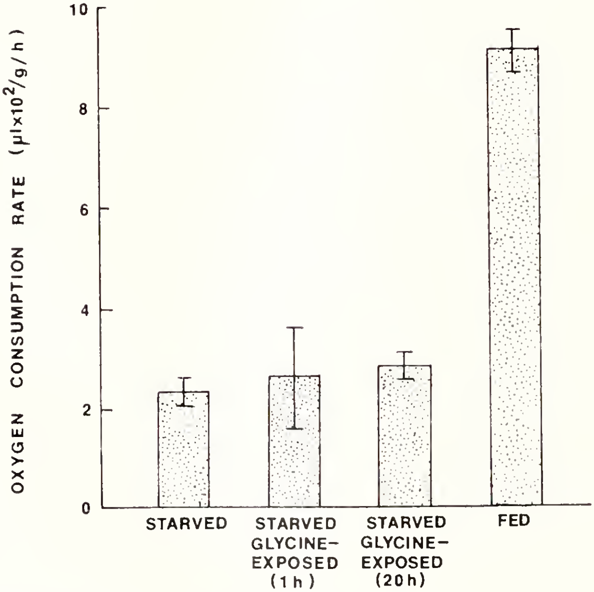


FIGURE 5. Oxygen consumption rates in 4 groups of 100–150 starved, starved/glycine-exposed (1 hr), and starved/glycine-exposed (20 hr) Texas polyps, and in 4 groups of 50 fed polyps. Values are means ± SD.

Free amino acid pools

Absolute amounts of acidic and neutral free amino acids (FAA) in pools of *Aurelia aurita* scyphistomae from Corpus Christi, Texas are presented elsewhere (Shick, 1974). The data show a general similarity to those for *Aurelia* from the York River, Virginia given by Webb, Schimpf and Olmon (1972), in that glycine and taurine predominate and that β-alanine is present.

Reliable wet tissue weights were not obtained, and values are necessarily expressed in terms of amount of amino acid per unit dry weight. While the concentrations of most FAA decline during 2 weeks of starvation, those of glycine, taurine and β-alanine increase from 89 to 154, 29 to 49, and 23 to 48 nmole/mg, respectively. The total FAA content (excluding undetermined basic amino acids) increases from 306 to 343 nmole/mg, although the actual *concentrations* may be more similar due to increased tissue hydration in the starved polyps.

DISCUSSION

This study provides direct evidence of a nutritional role of dissolved amino acids at environmentally realistic concentrations in starved *Aurelia aurita* scyphistomae. In addition, substantial data regarding temperature effects on the uptake of glycine, and on the kinetics of uptake, are presented.

The temperature sensitivity of glycine uptake by Texas *Aurelia* polyps (Fig. 2) is similar to that of other rate processes in warm-temperate zone scyphozoans, for example, the pulsation rate-temperature curve for *Aurelia* ephyrae given in Mangum, Oakes and Schick (1972), the pulsation R-T curve for *Chrysaora quinquecirrha* medusae in Gatz, Kennedy and Mihursky (1973), and the curves for *Aurelia* and *Cassiopea xamachana* medusae presented by Mayer (1914). It must be noted that the pulsation rates in *Aurelia* ephyrae and medusae, and in *Cassiopea* medusae, were acutely determined; only in the studies on *Chrysaora* medusae and in the present investigation of *Aurelia* polyps were the animals acclimated to the experimental temperatures.

Of considerable interest is the pattern of inverse temperature compensation of glycine uptake shown by the Texas polyps (Fig. 3A). Such an observation is not unprecedented, having also been noted in the uptake of solutes by bacteria and yeasts (Christophersen, 1967), and in the accumulation of ninhydrin positive substances by *Mya arenaria* (DuPaul and Webb, 1970). The latter work, however, involved apparently endogenously-derived materials accumulated by adductor muscle in response to increased salinity.

While Virginia polyps exhibit positive thermal acclimation of metabolic rate (Mangum *et al.*, 1972), they also show a pattern of inverse compensation of glycine uptake (Fig. 3B), similar to that of the Texas polyps, and the inverse compensation is therefore apparently not a latitudinal phenomenon. The inverse compensation is not necessarily maladaptive. On the contrary, since seasonal acclimation of oxygen consumption and other metabolic processes is less than perfect in scyphozoan polyps and medusae (*i.e.*, Q_{10} values > 1.0 [Mangum *et al.*, 1972; Gatz *et al.*, 1973]), it is advantageous for the warm acclimated animals to have an enhanced rate of metabolite transport.

The increasing affinity of the uptake system for glycine, indicated by the decreasing K_t values, as the acclimation temperature is lowered (Table II) would seem to be an example of seasonal "positive thermal modulation" as discussed by Hochochka and Somero (1973) for enzyme kinetics. If one accepts the premise that the uptake of glycine is mediated by a membrane transport protein or "enzyme" (for reviews, see: Stein, 1967; Pardee, 1968), then the decreasing K_t values with decreasing acclimation temperature may indeed be analogous to the phenomenon of positive thermal modulation described for enzyme kinetics.

From the results of the temperature acclimation experiments already discussed, however, it is obvious that the entire process of glycine uptake is not strictly analogous to that of enzyme activity. The reasons for the seeming discrepancy between inverse temperature compensation of glycine uptake and positive thermal modulation of the affinity of the uptake system are likely to be numerous, but essentially rest in the fact that the experiments were performed on intact animals. While the transport system exhibits increasing affinity for glycine as the temperature is lowered, the total influx of glycine is differently affected, being greater in the warm acclimated polyps, as evidenced by the inverse temperature compensation and by the large temperature effect on V_{max} (Table II). This may result from a reduced number of available transport sites, due to changes in membrane lipid composition, or from a diminished turnover of glycine by the carrier, in the cold acclimated polyps.

Previous investigators have discussed variation of K_t values for amino acid uptake by marine invertebrates almost exclusively with reference to the animals' adaptations to the environmental free amino acid concentrations to which they are normally exposed (Southward and Southward, 1972a, 1972b; Stephens, 1972; Dixit, 1973). The K_t value for glycine uptake by *Aurelia* ephyrae is essentially the same as that of the polyps at the same temperature (Table II). If the environmental FAA concentration is the principal determining factor for the affinity of the amino acid uptake system, it would appear that the polyp, which is epifaunal on a variety of firm substrates, and the ephyra, an actively swimming plankter, are adapted to the same environmental glycine concentration. The microstratification of dissolved organic matter is poorly understood; however, the difference in DFAA concentration is likely to be greater between the interstitial water and that at the sediment surface than among the latter and different levels in the water column. Analyses of water from different microhabitats are certainly necessary to clarify this point.

While the above consideration is no doubt important and perhaps ultimately the prime determinant, the effect of environmental temperature on K_t values has not been considered heretofore. The present study has demonstrated a significant temperature effect on K_t values for glycine uptake by *Aurelia* polyps. The values at the lower acclimation temperatures are within the range of K_t values for amino acids given by Southward and Southward (1970, 1972a, 1972b) for deep sea pogonophorans. At the highest temperature they extend within the range of K_t values for amino acid uptake by a variety of marine and estuarine invertebrates summarized by Stephens (1967) and Dixit (1973). The K_t value for glycine uptake by *Chrysaora quinquecirrha* scyphistomae is 19 μM (K. L. Webb, personal communication), at the middle of the range of values reported for *Aurelia* polyps in the present investigation. The K_t values summarized by Stephens and by

Dixit, and that reported by Webb, were determined at or near room temperature; the experiments by the Southwards were performed at 4°–6° C, and the K_t's which they reported would presumably increase at higher (although unnatural for their specimens) temperatures. It becomes evident that intra- and interspecific comparisons of affinities of amino acid uptake systems based solely on known environmental DFAA concentrations may permit only an incomplete understanding of the implications of the process, since a temperature effect on K_t is an additional modifying factor in eurythermal invertebrates.

The results of several lines of experimentation demonstrate that dissolved glycine, at the environmentally realistic concentration of 0.80 μM, is a supplemental nutritional source for starved *Aurelia aurita* scyphistomae.

While polyps of *Aurelia* are known to be resistant to prolonged starvation (Spangenberg, 1967), the degree of nutritive preparation has a demonstrably profound effect on strobilation (Thiel, 1962; Spangenberg, 1967; Russell, 1970). The results summarized in Table V indicate that starvation of Texas polyps for 56 days markedly reduces the incidence of strobilation. Of greater import to the present discussion is the observation that this diminution of the number of animals strobilating can be abolished by exposing the polyps to environmental concentrations of glycine or alanine during the period of food deprivation. Additional results in Table V, as well as qualitative differences in strobilation (developmental anomalies, meristic variation in ephyrae, *etc.*) between starved and starved/amino acid-exposed polyps, further demonstrate that dissolved amino acids are indeed a supplemental nutritional source for these animals during 56 days of starvation in the laboratory.

It therefore seemed particularly worthwhile to determine whether the importance of these molecules is as a source of reduced carbon for energy-yielding metabolism (*i.e.,* as a "supplemental energy source"), or of nitrogen for amino and nucleic acids, or whether a combination of factors is involved. Accordingly, subsequent experiments were performed in which polyps were given dissolved glucose during the 8-week food deprivation period. The concentration was adjusted so that polyps in this group had approximately the same amount of carbon available to them as did the starved/glycine-exposed polyps, the principal difference being the presence or absence of exogenous nitrogen.

Most importantly, it was found that the diminution of the strobilation response in starved animals can also be abolished by their exposure to dissolved glucose. Thus, both nitrogenous and non-nitrogenous substrates may exert a sparing effect on endogenous polyp materials, which are converted to ephyra materials during metamorphosis.

Serious developmental anomalies and meristic variation occur with a high frequency in starved polyps and in those receiving glucose, but to a much lesser extent in starved/amino acid-exposed and in fed animals (Table V). That exogenous nitrogen sources are necessary for normal metamorphosis and development is therefore obvious, especially when one recalls that the experimental animals are genetically identical. The demonstrated 41–260% increases in DNA per polyp and probable continuous synthesis of RNA and protein during strobilation (Black, 1972) further emphasize the importance of nitrogenous materials during this period, and may give some insight as to at what levels the dissolved organic nitrogen sources in the present experiment exert their effects.

The results of this entire line of experimentation indicate that the polyp-to-ephyra transformation is of fundamental importance in *Aurelia aurita*, and once induced and initiated, proceeds to its conclusion even under severe nutritive stress. These results may also help to explain a variety of well known observations on *Aurelia*. Developmental anomalies and meristic variation have been studied in *Aurelia* medusae and ephyrae at least since 1837 (see discussions in Berrill, 1949; Thiel, 1959; and Russell, 1970). While the possibility remains that much of the observed morphological variation is due to genetic differences among individuals and to undetermined environmental factors, the present investigation, using clonal polyps, has demonstrated that nutritional conditions play an extensive role in producing such variations. The production of both normal and abnormal (specifically, those having more than 8 lobes) ephyrae by the same well fed strobila, also discussed by Berrill (1949) remains unexplained, although it is apparently a fairly common occurrence.

Exposure of starved polyps to dissolved organic compounds has no effect on the process of budding. All groups of starved polyps, whether or not they were exposed to these compounds, ceased budding after 14–15 days of food deprivation. This cessation is reflected in the decreased percentage incorporation of labeled glycine into ethanol insoluble materials by starved animals (Table III, and Shick, 1973). It would not be advantageous for a population of relatively sessile polyps to increase its size, and hence its demand for food, during periods of food scarcity. If dissolved amino acids and other organic molecules have a supplemental nutritional role or sparing effect on developmental processes, this role is more likely to be manifested in the process of strobilation, which provides a means for animals to leave the local population (*i.e.*, by swimming away as ephyrae), and assures dispersal of the sexual stage of the life cycle.

Aurelia ephyrae have a higher weight-specific V_{max} for glycine uptake than do the polyps (Table II). It is unknown whether this is due to the smaller size of the former, to their proportionally greater surface area, or to an increase in the number of carrier sites or turnover of glycine. Whichever is the case, the importance of a greater uptake rate to the ephyra, a planktonic larval stage with limited nutrient reserves, yet with a much higher activity level and energy demand than the sessile polyp (Mangum *et al.*, 1972), seems obvious.

Oxygen consumption by marine invertebrates is known to decline during food deprivation (Roberts, 1957; Vernberg, 1959; Thompson and Bayne, 1972; Bayne, 1973a, 1973b; Newell, 1973; Wallace, 1973; and others), although the time course over which this occurs is extremely variable among the organisms studied. Oxygen consumption decreases to 25.9% of the value in fed polyps in *Aurelia* scyphistomae starved for 14 days (Fig. 5).

That oxygen consumption increases significantly in starved polyps following exposure to environmental concentrations of dissolved glycine (Fig. 5) may further indicate an energy-providing role of dissolved glycine. While the total glycine flux in fed and starved polyps remains undetermined, such an activation of catabolic pathways does not seem likely if there is a net loss of material. The possibility that the increase is a reflection of the initiation of the feeding response by glycine (Loeb and Blanquet, 1973) cannot be discounted, although the persistence of a higher rate of oxygen consumption after termination of 20 hr of glycine exposure would seem to argue against it.

Qualitative and quantitative analyses of the FAA pools of fed and starved polyps reveal that while the concentrations of most free amino acids decline during 2 weeks of food deprivation, there is little change in the internal FAA concentration. This concentration is maintained largely by increases in glycine, taurine and β-alanine. These amino acids are the principal constituents of the pools of the starved scyphistomae, and are known to be important in the total osmotic concentration of the tissues of other marine invertebrates. Their increased concentrations in starved polyps may be a compensatory mechanism to maintain the internal concentration of osmotically active substances during the utilization of other amino acids in the pool. The presence of β-alanine in the Texas scyphistomae is also significant from the standpoint of biochemical divergence among geographically separated *Aurelia aurita* populations (Webb *et al.,* 1972; Morales-Alamo and Haven, 1974; Shick, 1974).

Since the internal free glycine concentration actually increases in polyps after 14 days of food deprivation, and since these animals take up the same amount of labeled glycine per unit body weight as do fed animals (Fig. 2), then the increase in $^{14}CO_2$ production by starved scyphistomae (Fig. 4) does in fact demonstrate an enhanced catabolism of glycine by these animals, as tentatively suggested by Shick (1973). The enhancement of glycine catabolism by starved polyps is due at least in part to the more complete breakdown of the glycine molecule by these animals, as indicated by the increased (doubled) appearance of alpha-carbon label in $^{14}CO_2$ (Table IV). The lag in $^{14}CO_2$ production by starved polyps, followed by its rapid appearance (Fig. 4), may be a further indication of the activation of catabolic pathways as suggested by the oxygen consumption determinations. Since the absolute and percentage glycine concentration rises considerably in starved polyps, and since the uptake of exogenous glycine would tend to accentuate this imbalance, the basis for the increased glycine catabolism may be the offsetting of such an imbalance.

The pathways of glycine catabolism in *Aurelia* polyps remain unclear. Glycine catabolism does not normally proceed via the citric acid cycle (for discussion, see Shick, 1973). In addition, unpublished personal observations suggest that while the occurrence of the glyoxylate cycle in *Aurelia,* as proposed by Raum (1970), cannot be discounted, it appears to be of little quantitative significance in glycine catabolism.

Despite the above facts, a large amount of $^{14}CO_2$ is produced from the alpha carbon of labeled glycine by *Aurelia* scyphistomae. It is now known that the oxidative glycine cleavage system of ammonotelic animals can produce CO_2 from the alpha carbon (Kikuchi, 1973) and this might account for the observations in the present study. The predominance of carboxyl-carbon radioactivity in the $^{14}CO_2$ produced by fed polyps (Table IV) indicates that the labeled glycine is broken down primarily via decarboxylation, perhaps in a glycine cleavage system, with the alpha carbon being channelled to the one-carbon pool for biosynthetic processes. The increased production of CO_2 from the alpha carbon by starved scyphistomae would provide additional reduced pyridine nucleotide for increased energy production in these polyps.

Assuming that glycine is catabolized via the glycine cleavage system and not via the citric acid cycle or glyoxylate cycle, then the complete breakdown of 1 mole of glycine would produce 1 mole each of $NADH_2$ and $NADPH_2$ (Kikuchi, 1973), which would require 1 mole of O_2 for reoxidation. The glycine uptake rate at 20° C from an ambient glycine concentration of 0.80 μM could then provide sufficient glycine to support approximately 6% of the observed oxygen consumption rate in starved/glycine-exposed polyps. Glycine, while calorically poor relative to other amino acids, is generally the most concentrated amino acid dissolved in seawater (Webb and Wood, 1966; Siegel, 1967; Hobbie, Crawford and Webb, 1968; Bohling, 1970; Andrews and Williams, 1971; Clark *et al.*, 1972). This fact, coupled with its increased oxidation during starvation, may enhance its importance as a supplemental energy source for marine invertebrates.

The long-term starvation experiments (Table V) also demonstrated the importance of glycine as a source of nitrogen. The glycine uptake rate in starved polyps at 20° C and 0.80 μM glycine could provide about 0.26 μg N per starved polyp (average dry weight 35 μg) during the 56-day starvation period. Assuming that roughly 1–3% of the dry weight of semaeostome scyphozoans is nitrogen (Vinogradov, 1953, pages 198–199), this means that starved/glycine-exposed polyps take up nitrogen equivalent to 25–74% of their total body nitrogen during the starvation period.

The above calculations do not take into consideration the amount of glycine released by the polyps through leakage or other means. Such information, while difficult to obtain accurately, is certainly necessary if the significance of glycine uptake is to be stated quantitatively.

It has become apparent that a full understanding of the significance of the uptake of dissolved amino acids must take into account a number of factors. While environmental temperature, salinity and DFAA concentration determine the magnitude and dynamics of the uptake of these compounds by the animal in question, nutritive state has a direct bearing on the allocation of these materials between catabolic and anabolic pathways. Comparisons among fed, starved and starved/amino acid-exposed *Aurelia aurita* scyphistomae have shown that dissolved amino acids are important in the alleviation of nutritive stress in these organisms, this importance being manifested in biochemical, physiological and developmental processes.

I thank J. W. Anderson and R. M. Darnell for their encouragement and material support during the course of this work. J. M. Prescott provided time on the amino acid analyzer. Appreciation is also extended to R. E. L. Black, C. P. Mangum and G. C. Stephens for critically reading an earlier draft of the manuscript.

This paper is adapted from a dissertation submitted to the Graduate College of Texas A&M University in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

SUMMARY

1. The temperature sensitivity of glycine uptake by *Aurelia aurita* scyphistomae from Corpus Christi, Texas is similar to that of other rate processes in warm-temperate zone scyphozoans.

2. Both Texas polyps and those from the York River, Virginia show inverse temperature compensation of glycine uptake; the phenomenon is therefore apparently not latitudinally based.

3. The values of K_t and V_{max} for glycine uptake are directly related to temperature between 12° and 32° C. The increasing affinity of the glycine uptake system with decreasing temperature may be analogous to "positive thermal modulation" of enzyme-substrate affinity in poikilotherms.

4. The fivefold increase in K_t between 12° and 32° C indicates that environmental temperature is an important consideration in intra- and interspecific comparisons of the affinities of amino acid uptake systems in marine invertebrates.

5. Eight weeks of food deprivation at 20° C result in a 77.5% reduction in the number of polyps strobilating in response to temperature increase and exposure to iodide. This effect can be abolished by exposing starved polyps to environmental concentrations of glycine or alanine during the starvation period.

6. Exposure of starved polyps to dissolved glucose during the 8-week period also overrides the diminution of the strobilation response. However, starved and starved/glucose-exposed polyps produce a higher percentage of abnormal ephyrae than do fed and starved/amino acid-exposed polyps, emphasizing the importance of dissolved amino acids as nitrogen sources.

7. All starved polyps, whether or not they are exposed to dissolved organic compounds, cease budding after 14–15 days of food deprivation.

8. Oxygen consumption declines to 25.9% of the value in fed polyps during 2 weeks of food deprivation. Exposure of starved polyps to dissolved glycine produces an increase in this parameter.

9. There is no effect of 2 weeks of food deprivation on glycine uptake by polyps. However, starvation does produce an enhanced rate of glycine catabolism, due in part to the increased production of CO_2 from the alpha carbon of the molecule. The predominance of glycine among amino acids dissolved in seawater, and its increased oxidation during starvation, may enhance its importance as a supplemental energy source for marine invertebrates.

10. The internal pool concentrations of most free amino acids decline during 2 weeks of food deprivation; the total FAA concentration of the pools is little affected, largely due to compensatory increases in glycine, taurine and β-alanine.

LITERATURE CITED

ANDREWS, P., AND P. J. LEB. WILLIAMS, 1971. Heterotrophic utilization of dissolved organic compounds in the sea. III. Measurements of the oxidation rates and concentrations of glucose and amino acids in sea water. *J. Mar. Biol. Ass. U.K.*, **51**: 111–125.

BAYNE, B., 1973a. Aspects of the metabolism of *Mytilus edulis* during starvation. *Netherlands J. Sea Res.*, **7**: 399–410.

BAYNE, B. L., 1973b. Physiological changes in *Mytilus edulis* L. induced by temperature and nutritive stress. *J. Mar. Biol. Ass. U.K.*, **53**: 39–58.

BERRILL, N. J., 1949. Developmental analysis of scyphomedusae. *Biol. Rev.*, **24**: 393–410.

Black, R. E., 1972. Nucleic acid and protein levels in strobilating polyps of *Chrysaora quinquecirrha* and *Aurelia aurita*. *Mar. Biol.*, **16**: 134–137.

Bohling, H., 1970. Untersuchungen über freie gelöste Aminosäuren in Meerwasser. *Mar. Biol.*, **6**: 213–225.

Bohling, H., 1972. Gelöste Aminosäuren in Oberflächenwasser der Nordsee bei Helgoland: Konzentrationsveränderungen im Sommer 1970. *Mar. Biol.*, **16**: 281–289.

Christophersen, J., 1967. Adaptive temperature responses of microorganisms. Pages 327–348 in C. L. Prosser, Ed., *Molecular Mechanisms of Temperature Adaptation.* American Association for the Advancement of Science, Washington, D. C.

Clark, M. E., 1964. Biochemical studies on the coelomic fluid of *Nephtys hombergi* (Polychaeta: Nephtyidae), with observations on changes during different physiological states. *Biol. Bull.*, **127**: 63–84.

Clark, M. E., G. A. Jackson and W. J. North, 1972. Dissolved free amino acids in southern California coastal waters. *Limnol. Oceanogr.*, **17**: 749–758.

Coast and Geodetic Survey, 1968. *Surface Water Temperature and Density, Atlantic Coast, North and South America.* Coast and Geodetic Survey Publication 31–1, Washington, D. C., 102 pp.

Coyne, J. A., 1973. An investigation of the dynamics of population growth and control in scyphistomae of the scyphozoan *Aurelia aurita*. *Chesapeake Sci.*, **14**: 55–58.

DiSalvo, L. H., 1971. Ingestion and assimilation of bacteria by two scleractinian coral species. Pages 129–136 in H. M. Lenhoff, L. Muscatine and L. V. Davis, Eds., *Experimental Coelenterate Biology.* University of Hawaii Press, Honolulu.

Dixit, D. B., 1973. Uptake of amino acids and development in the sea urchin, *Strongylocentrotus purpuratus*. *Ph.D. dissertation, University of California, Irvine,* 117 pp.

DuPaul, W. D., and K. L. Webb, 1970. The effect of temperature on salinity-induced changes in the free amino acid pool of *Mya arenaria*. *Comp. Biochem. Physiol.*, **32**: 785–801.

Gatz, A. J., Jr., V. S. Kennedy and J. A. Mihursky, 1973. Effects of temperature on activity and mortality of the scyphozoan medusa, *Chrysaora quinquecirrha*. *Chesapeake Sci.*, **14**: 171–180.

Hobbie, J. E., C. C. Crawford and K. L. Webb, 1968. Amino acid flux in an estuary. *Science*, **159**: 1463–1464.

Hochachka, P. W., and G. N. Somero, 1973. *Strategies of Biochemical Adaptation.* Saunders, Philadelphia, 358 pp.

Johannes, R. E., S. J. Coward and K. L. Webb, 1969. Are dissolved amino acids an energy source for marine invertebrates? *Comp. Biochem. Physiol.*, **29**: 283–288.

Kikuchi, G., 1973. The glycine cleavage system: Composition, reaction mechanism, and physiological significance. *Mol. Cell. Biochem.*, **1**: 169–187.

Loeb, M. J., and R. S. Blanquet, 1973. Feeding behavior in polyps of the Chesapeake Bay sea nettle, *Chrysaora quinquecirrha* (Desor, 1848). *Biol. Bull.*, **145**: 150–158.

Mangum, C. P., M. J. Oakes and J. M. Shick, 1972. Rate-temperature responses in scyphozoan medusae and polyps. *Mar. Biol.*, **15**: 298–303.

Mayer, A. G., 1914. The effects of temperature upon tropical marine animals. *Pap. Tortugas Lab.*, 6: 1–24.

Morales-Alamo, R., and D. S. Haven, 1974. Atypical mouth shape of polyps of the jellyfish, *Aurelia aurita*, from Chesapeake Bay, Delaware Bay, and Gulf of Mexico. *Chesapeake Sci.*, **15**: 22–29.

Newell, R. C., 1973. Factors affecting the respiration of intertidal invertebrates. *Amer. Zool.*, **13**: 513–528.

Pardee, A. B., 1968. Membrane transport proteins. *Science*, **162**: 632–637.

Percival, E., 1923. On the strobilization of *Aurelia*. *Quart. J. Microscop. Sci.*, **67**: 85–100.

Prosser, C. L., 1964. Perspectives of adaptation: Theoretical aspects. Pages 11–25 in D. B. Dill, E. F. Adolph and C. G. Wilber, Eds., *Handbook of Physiology.* Section 4. American Physiological Society, Washington, D. C.

Raum, W. J., 1970. Glycine metabolism in the scyphistoma of *Aurelia aurita*. *M.A. thesis, Bowling Green State University*, 21 pp.

Roberts, J. L., 1957. Thermal acclimation of metabolism in the crab *Pachygrapsus crassipes*

Randall. I. The influence of body size, starvation, and molting. *Physiol. Zoöl.*, **30**: 232–242.

RUSSELL, F. S., 1970. *The Medusae of the British Isles, Vol. 2, Pelagic Scyphozoa with a Supplement to the First Volume on Hydromedusae.* The University Press, Cambridge, 284 pp.

SCHLICHTER, D., 1973. Ernährungsphysiologische und ökologische Aspekte der Aufnahme in Meerwasser gelöster Aminosäuren durch *Anemonia sulcata* (Coelenterata, Anthozoa). *Oecologia*, **11**: 315–350.

SHICK, J. M., 1973. Effects of salinity and starvation on the uptake and utilization of dissolved glycine by *Aurelia aurita* polyps. *Biol. Bull.*, **144**: 172–179.

SHICK, J. M., 1974. Uptake and utilization of dissolved glycine by *Aurelia aurita* scyphistomae: Temperature effects on the uptake process; nutritional role of dissolved amino acids. *Ph.D. dissertation, Texas A&M University*, 87 pp.

SIEGEL, A., 1967. A new approach to the concentration of trace organics in seawater. Pages 235–256 in T. A. Olson and F. J. Burgess, Eds., *Pollution and Marine Ecology*. Wiley-Interscience, New York.

SOROKIN, YU. I., 1973. On the feeding of some scleractinian corals with bacteria and dissolved organic matter. *Limnol. Oceanogr.*, **18**: 380–385.

SOUTHWARD, A. J., AND E. C. SOUTHWARD, 1970. Observations on the role of dissolved organic compounds in the nutrition of benthic invertebrates. Experiments on three species of Pogonophora. *Sarsia*, **45**: 69–96.

SOUTHWARD, A. J., AND E. C. SOUTHWARD, 1972a. Observations on the role of dissolved organic compounds in the nutrition of benthic invertebrates. II. Uptake by other animals living in the same habitat as pogonophores, and by some littoral Polychaeta. *Sarsia*, **48**: 61–70.

SOUTHWARD, A. J., AND E. C. SOUTHWARD, 1972b. Observations on the role of dissolved organic compounds in the nutrition of benthic invertebrates. III. Uptake in relation to organic content of the water. *Sarsia*, **50**: 29–46.

SPANGENBERG, D. B., 1964. New observations on *Aurelia*. *Trans. Amer. Microscop. Soc.*, **83**: 448–455.

SPANGENBERG, D. B., 1967. Iodine induction of metamorphosis in *Aurelia*. *J. Exp. Zool.*, **165**: 441–450.

STEIN, W. D., 1967. *The Movement of Molecules across Cell Membranes.* Academic Press, New York, 369 pp.

STEPHENS, G. C., 1962a. Uptake of amino acids by the bamboo worm, *Clymenella torquata*. *Biol. Bull.*, **123**: 512.

STEPHENS, G. C., 1962b. Uptake of organic material by aquatic invertebrates. I. Uptake of glucose by the solitary coral, *Fungia scutaria*. *Biol. Bull.*, **123**: 648–659.

STEPHENS, G. C., 1967. Dissolved organic material as a nutritional source for marine and estuarine invertebrates. Pages 367–373 in G. H. Lauff, Ed., *Estuaries*. American Association for the Advancement of Science, Washington, D. C.

STEPHENS, G. C., 1972. Amino acid accumulation and assimilation in marine organisms. Pages 155–184 in J. W. Campbell and L. Goldstein, Eds., *Symposium on Nitrogen Metabolism and the Environment.* Academic Press, New York.

THIEL, HJ., 1962. Untersuchungen über die Strobilisation von *Aurelia aurita* Lam. an einer Population der Kieler Förde. *Kieler Meeresforsch.*, **18**: 198–230.

THIEL, M. E., 1959. Scyphomedusae. Semaeostomeae, Physiologie. *Bronn's Kl. Ordn. Tierreichs 2, Abt. II*, **2**(6): 849–1072.

THOMPSON, R. J., AND B. L. BAYNE, 1972. Active metabolism associated with feeding in the mussel *Mytilus edulis* L. *J. Exp. Mar. Biol. Ecol.*, **9**: 111–124.

VERNBERG, F. J., 1959. Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus *Uca*. II. Oxygen consumption of whole organisms. *Biol. Bull.*, **117**: 163–184.

VINOGRADOV, A. P., 1953. *The Elementary Chemical Composition of Marine Organisms.* Memoir 2, Sears Foundation for Marine Research, Yale University, New Haven, 647 pp.

WALLACE, J. C., 1973. Feeding, starvation and metabolic rate in the shore crab *Carcinus maenas*. *Mar. Biol.*, **20**: 277–281.

WEBB, K. L., A. L. SCHIMPF AND J. OLMON, 1972. Free amino acid composition of scyphozoan polyps of *Aurelia aurita, Chrysaora quinquecirrha* and *Cyanea capillata* at various salinities. *Comp. Biochem. Physiol.*, **43B**: 653–663.

WEBB, K. L., AND L. WOOD, 1966. Improved techniques for analysis of free amino acids in seawater. Pages 440–444 in *Automation in Analytical Chemistry,* Technicon Symposia, Vol. 1. Mediad, White Plains, New York.

WIEBE, W. J., 1971. Perspectives in microbial ecology. Pages 484–497 in E. P. Odum, *Fundamentals of Ecology.* [3rd Ed.] Saunders, Philadelphia.

WONG, L., 1971. Uptake of amino acids by marine annelids. I. Mechanisms of uptake and rate of loss. II. Regulatory control and effect on free amino acid pool. *Ph.D. dissertation, University of California, Irvine,* 130 pp.

ZOBELL, C. E., AND C. B. FELTHAM, 1938. Bacteria as food for certain marine invertebrates. *J. Mar. Res.,* **1**: 312–327.

Reference: *Biol. Bull.*, **148**: 141–156. (February, 1975)

HISTOFLUORESCENT LOCALIZATION OF SEROTONIN AND DOPAMINE IN THE NERVOUS SYSTEM AND GILL OF *MYTILUS EDULIS* (BIVALVIA)[1]

GEORGE B. STEFANO AND EDWARD AIELLO

Department of Biological Sciences, Fordham University, Bronx, New York 10458

In bivalve molluscs there has been physiological evidence of neural control of ciliary activity (Koshtoyants, Buznikov and Manukhin, 1961; Aiello and Guideri, 1964, 1966; Takahashi and Murakami, 1968). There has also been histological evidence for the innervation of ciliated gill filaments (Splittstosser, 1913; Setna, 1930; Aiello and Guideri, 1965; Sweeney, 1968). Specifically, in *Mytilus edulis*, Aiello and Guideri (1965) have reported that nerve fibers from the branchial nerve enter individual gill filaments and run beneath the lateral cells. An electron micrographic description of this innervation has been given by Paparo (1972). Sweeney (1968) has reported the presence of nerves in the gill filament of *Sphaerium sulcatum*. Grave and Schmitt (1925) reported a bipolar nerve cell system beneath the ciliated epithelium of the gill in the bivalves *Lampsilis*, *Anodonta* and *Mya*.

It has also been established in *M. edulis* that transection of the branchial nerve depresses ciliary activity and this activity was increased by electrical stimulation in the intact animal or in the gill-nerve-visceral ganglion preparation (Aiello and Guideri, 1964, 1965; Takahashi and Murakami, 1968).

Other experimenters have proposed the concept of local hormonal control employing acetylcholine (Bulbring, Burn and Shelly, 1953) or serotonin (Gosselin, Moore and Milton, 1962). Acetylcholine has been found to be both a positive and a negative modulator of the frontal cilia and this effect is concentration dependent (Bulbring *et al.*, 1953; Aiello and Paparo, 1974). However, acetylcholine esterase activity is largely localized in the nervous system (Bouffard, 1970) and the response of cilia on the gill to electrical stimulation of the branchial nerve is altered by the administration of physostigmine, a cholinesterase inhibitor (Aiello and Paparo, 1974). One might equally well, therefore, attribute to endogenous acetylcholine the role of neurotransmitter, with possible actions both on ciliated cells and on elements of the nervous system.

Serotonin was also found to have a cilio-excitatory and metabolic stimulatory effect in several lamellibranch gills, and may be an endogenous regulator (Gosselin *et al.*, 1962; Schor, 1965; Sweeney, 1968; Aiello, 1970). Its precursors, tryptophan and 5-hydroxytryptophan have been found in the gill of *M. edulis* (Aiello, 1960, 1962; Gosselin *et al.*, 1962), and the conversion of 5-hydroxytryptophan to serotonin and further breakdown products has been demonstrated (Blaschko and Milton, 1960; Aiello, 1965). It is not known exactly where these substances are located but Sweeney (1968) has demonstrated the occurrence of serotonin and some catecholamine in gill filaments of *S. sulcatum* by the histochemical fluorescence technique.

---

[1] This work was supported in part by a grant from the U.S.P.H.S. (ND-07402).

A second monoamine implicated in the regulation of ciliary activity is dopamine. The administration of dopamine to the lateral cilia results in a cilioinhibition (Paparo and Aiello, 1970). Dopamine has been found in the nervous system of a number of bivalve molluscs (Dahl, Falck, Lindquist and Von Mecklenburg, 1962; Dahl, Falck, Von Mecklenburg, Myhrberg and Rosengren, 1966; Sweeney, 1963, 1968) and in extracts of bivalve gills (Malanga, Wenger and Aiello, 1972).

In *M. edulis* the only report of ganglionic morphology was published in 1887 by Rawitz. He concluded that histologically the ganglia of *M. edulis* follow the typical pattern for Pelecypods, having a cortex surrounding a central neuropile and containing no large cell bodies or fibers (Rawitz, 1887). Localization of monoamines in invertebrates became possible with the advent of the histochemical fluorescent technique of Falck, Hillarp, Thieme and Torp (1962). Various investigators have used this technique successfully in bivalves (Dahl *et al.*, 1966; Zs.-Nagy, 1968; Sweeney, 1968).

The purpose of this research was to see if the localization of the monoamines serotonin and dopamine is correlated with the control of ciliary activity. The physiological and pharmacological evidence cited above seems to call for the dual innervation of the ciliated epithelium of *M. edulis* by serotonin and dopamine containing neurons. It was also of interest to know more about the central nervous system itself such as the distribution and interrelationship of specific neurons in the ganglia and the location of cell bodies of neurons innervating the gill in the hope of being able to relate this information to an understanding of ciliary control. A preliminary report of some of this work has already been presented (Stefano and Aiello, 1974).

Materials and Methods

Mytilus edulis was obtained from two sources: some animals were harvested from rocks in the intertidal zone of Pelham Bay, Long Island Sound, New York and some animals were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. The animals were kept in artificial seawater (Instant Ocean Aquarium System) at 19° C, pH 7.5–7.8 and specific gravity 1.025.

Three criteria were used in selecting a healthy animal for experimentation: (1) it closed its shell quickly when the siphon was touched; (2) when the foot in the gaping animal was touched, it reflexively withdrew it and then closed the valves; (3) the valves remained closed against a reasonable amount of force applied to open them.

Treatment of whole animals with chemical agents was accomplished either by injection or by direct exposure in the bathing medium. For injection, the valves were opened with a retractor and injection made into the foot or the posterior adductor muscle and sometimes in both. The animal was then placed in a liter beaker with 300 milliliters of seawater and its own oxygen source, but maintained at the original temperature by immersing the beaker in the main aquarium. The same system was utilized for direct exposure, except that the chemical agent was dissolved in the bathing medium.

The valves were forced open and the posterior adductor muscle cut midway between the left and right visceral ganglia. The cerebral ganglia were removed first. This was done by placing a relatively thick piece of paper under them and pinning tissue around the ganglia to the paper. Then the tissue and paper was cut free and immersed in isopentane cooled by liquid nitrogen to about −170° C. The visceral ganglion was removed with a piece of supporting posterior adductor muscle and frozen as above. In some instances the visceral ganglion and associated gill were removed together for further experimentation.

Frozen tissues were transferred rapidly to the thermal plate of an Edwards-Pearse Tissue Freeze Dryer and dried for five days at a temperature of −45° C and a vacuum of 10^{-3} torr.

After this drying period the tissues were gradually brought to room temperature and the vacuum broken by the introduction of dry nitrogen into the chamber. The tissues were rapidly transferred to a steel grid over dry paraformaldehyde in a small, wide-mouthed jar. The paraformaldehyde was routinely stored *in vacuo* over phosphorus pentoxide at room temperature. This jar was then sealed by a screw cap, placed in preheated oven and kept at a temperature of 80° C for 1.5 hrs.

Paraffin to be used for embedding was melted at 56° C and degassed in a vacuum. Small amounts were further degassed in a container on the thermal plate of the tissue drier and allowed to cool. A pit was dug into the wax and the dry, paraformaldehyde-treated tissue was placed in the pit. The tissue was infiltrated in a vacuum at 60° C for approximately one hour or until bubbles ceased to appear from the tissue, which was never more than two hours. The vacuum was then broken and the tissue transferred to fresh paraffin for about four hours at 60° C, then placed in plastic embedding blocks and solidified at room temperature.

Tissues were sectioned on a standard microtome. The sections were mounted on non-fluorescent slides in Fluoromount (Fisher) containing 10% dry xylene or in dry xylene alone and flattened on a hot plate at 32° C.

Fluorescence was observed with a Reichert Zetopan fluorescent microscope fitted with Mercury Lamp HBO 200 W and either bright field or dark field Reichert condensors. The excitation filters BG-12 permitted activation at 410 nm to 440 nm, and UV-blue excluding filters GG9 and GG1 permitted the passage of green fluorescence from catecholamine and yellow fluorescence from serotonin reaction products (Marsden and Kerkut, 1969; Falck *et al.*, 1962; Falck and Owman, 1965). After observing fluorescence the slide was left in place and the optics changed to phase contrast as an aid in identifying the fluorescent structures. Some sections were stained with Harris hematoxylin and eosin. Other tissues were treated with Ramon y Cajal's silver-pyridine method (Favorsky, 1930).

A single lens reflex camera (Minolta SRT 101) was utilized for photomicroscopy. Color photography was done with High Speed Ektachrome film (ASA 164) and processed commercially. Black and white photography was done with Tri-x film (ASA 400) developed in Kodak D76 and printed on Kodak Polycontrast F or Kodabromide F-2 paper.

Autofluorescence was differentiated from specific fluorescence by comparing paraformaldehyde treated tissue to untreated tissue; fluorescence common to both was termed autofluorescence. Yellow autofluorescence was further differentiated from specific serotonin fluorescence in that the fluorophore of serotonin fades rapidly while autofluorescence remains. A specific test developed by Corrodi, Hillarp and Jonsson (1964) was also used in which the section on the slide was partially hydrated and treated with 0.03% sodium borohydride for two minutes and then re-examined under the fluorescence microscope. Autofluorescence remained but the specific fluorescence was reduced by sodium borohydride to a non-fluorescent product. Specific fluorescence was regained by resubjecting the slide to the paraformaldehyde treatment.

Observation and Results

Gross anatomy

The structures in the nervous system of *Mytilus edulis* examined in this investigation were the cerebral and visceral ganglia, the cerebrovisceral connective and the branchial nerve and its subdivisions. Our description is in essential agreement with that of Field (1922) except that he could not trace nerve fibers from the branchial nerve into the individual gill filaments. All indications are that *M. edulis* is bilaterally symmetrical throughout the animal and that our descriptions apply equally well to either side.

The cerebral ganglia are narrow bodies with their long apices pointing posteriorly. They lie on the ventral side of the esophagus 4 to 6 mm apart and united by a commissure. Each ganglion is about 2 mm in length and 1 mm wide at its base. The thickness varied but appeared to be approximately 0.5 mm at most. In some specimens, there was an orange-red pigment on its outer ventral surface of each ganglion.

The posterior trunk of each cerebral ganglion passes backward and outward across the ventral side of the anterior retractor muscle. At the lateral side of the anterior retractor muscle the trunk divides into two separate components, the cerebrovisceral and the cerebropedal connectives. The cerebrovisceral connective turns upward and continues in a posterior direction traveling along the lateral surfaces of the posterior retractor muscles and terminating in the visceral ganglion on that side.

The visceral ganglia are situated on the anterior ventral surface of the posterior adductor muscle close to the area where the gills are suspended. Each ganglion gives rise to several nerves. The branchial nerve leaves the posterior lateral side, runs obliquely ventrally and posteriorly to the base of the gill and sends a large number of fine fibers into the gill axis on that side of the animal.

Histology, cells of the cortex

Tissues stained by the standard hematoxylin and eosin procedure revealed the typical structural organization of the ganglia (Fig. 1). The largest nerve cell bodies were approximately 15 μ in diameter but the majority tended to be about 9–11 μ in diameter. There were also various types of smaller cells situated around the larger ones. The nerve cell bodies in both the cerebral and visceral ganglia were found to contain a granular cytoplasm with many inclusions, the largest of which were approximately 1 μ in diameter. The nerve cells were almost exclusively unipolar, having an approximately spherical cell body and a single gradually tapering cell process. Through the use of Ramon y Cajal's silver-pyridine method, which stained fine fibers, varicosities, and endings with a darker intensity, the cortex was found to be an area rich in terminal varicosities.

In general, the cells of the cortex appeared to be concentrated toward the ganglion's ventral surface. They were not uniformly distributed concentrically within the cortex, but were grouped into clusters.

Neuropile region

In both the visceral and cerebral ganglia a few nerve cell bodies were found in the neuropile region. These nerve cell bodies appeared to be extensively innervated as indicated by the specific staining of endings on their surface. The silver-pyridine method clearly showed how the process of the nerve cell body tapered as they extended from the body. The neuropile region offered a tangled appearance so that following an individual fiber for more than 15 μ was impossible. The fibers in the neuropile, however, did appear to possess some organization as shown by the presence of nerve tracts.

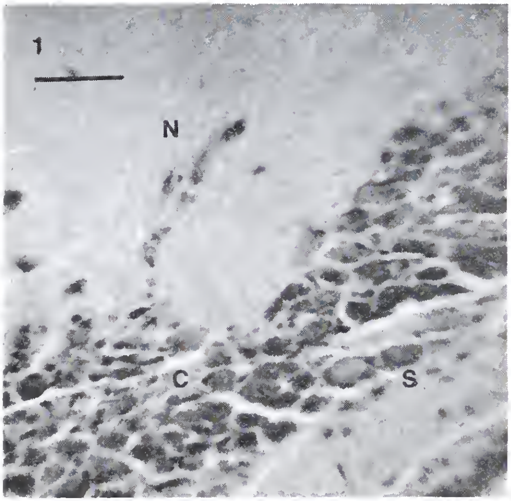
Connective tissue sheath

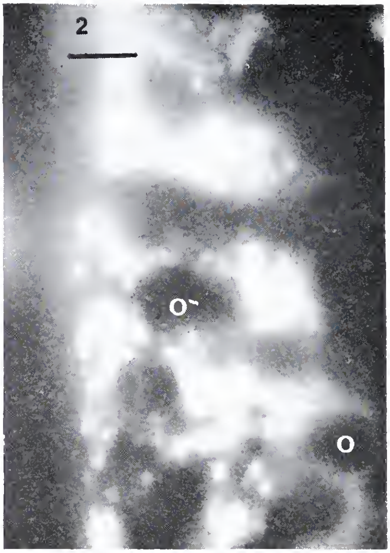
The connective tissue sheath in *M. edulis* not only covered the ganglia but also the connectives and nerves which emanated from the ganglia. Nerve cell bodies of the ganglia continued into the beginnings of the nerves originating from the ganglia, and these were found to be especially close to the sheath. By the use of the silver-pyridine method, nerve fibers were shown to be present in the sheath itself. No muscle fibers were observed there.

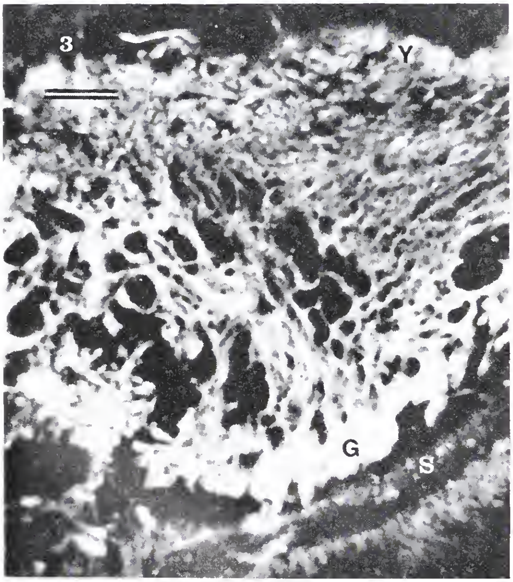
Fluorescent structures in the cerebral ganglion

In the cerebral ganglion specific yellow fluorescence was found in nerve cell bodies of 9 to 14 μ diameters located in the cortex (Fig. 2). Many of these cells were radially oriented and closely associated with the connective tissue sheath but a few were scattered throughout the cortex. The sheath had within itself yellow fluorescing granules, most of which seemed to be connected by fine yellow fibers, thereby constituting a network of varicose fibers (Fig. 2). Many of the nerve cell bodies in the ganglion cortex did not fluoresce (Fig. 2). In the neuropile region of the cerebral ganglion there were no yellow fluorescing cell bodies, but there were yellow granules, which gave the appearance of beads or varicose fibers. The distinction between granules and cell bodies had to be made under a 100 ×, oil immersion lens. There were also fine, yellow, varicose fibers in both the neuropile and the cortex.

The cerebral ganglion also contained green fluorescing nerve cell bodies (Fig. 3) which for the most part were slightly smaller in diameter than those emitting yellow fluorescence (Fig. 2). They were found in the cortex but not as close to the connective tissue sheath as the yellow fluorescing cells. The green fluorescence was not restricted to small intracellular granules but appeared to be homogeneous in its distribution (Fig. 3). The neuropile was extremely rich in green fluorescing fibers.







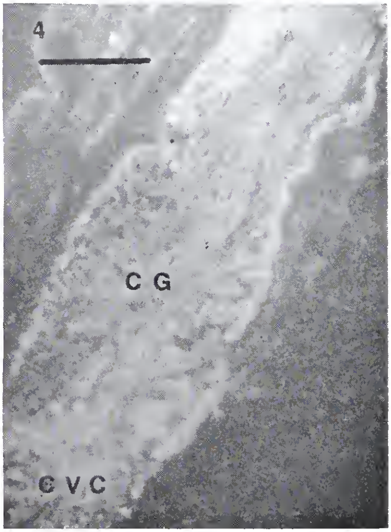


FIGURE 1. Hematoxylin and eosin stained section, 7 μ thick, of the cerebral ganglion showing representative appearance of neuropile (N), cortex (C) and thin connective tissue sheath (S). Scale bar = 10 μ.

FIGURE 2. Fluorescent photomicrograph of outer-part of the sheath and cortex of the cerebral ganglion showing cells packed with yellow fluorescing granules interspersed with non-fluorescing cells (O); (cells were identified by phase contract microscopy of same preparation). The sheath contains yellow fluorescing fibers and granules of similar appearance but no cells. Scale bar = 5 μ.

FIGURE 3. Cross section of visceral ganglion showing groups of green fluorescing cells (G) clustered at the periphery, especially on the ventral side (bottom of picture). High magnification never reveals discrete granules, as with yellow fluorescing cells (Y), but homogoneous green fluorescence. A few granules and fibers of the neuropile are yellow, the

Fluorescent structure in the visceral ganglion

The visceral ganglion contained only a few yellow fluorescing nerve cell bodies, these being 11 to 14 μ in diameter. Some yellow fibers were associated with both the cerebrovisceral connective and the branchial nerve. Yellow fibers were also seen in the predominantly green cortical region and neuropile along with yellow fluorescent beads previously described. The majority of the beads were present in the neuropile, while only a few were in the cortex.

The visceral ganglion cortex contained many green fluorescing cell bodies (Fig. 4) most of which were 10–12 μ in diameter and exhibited a homogenous green fluorescence. Processes could be seen to emanate from the nerve cell bodies. Green fibers could be seen in the cerebrovisceral connective and in the origin of the branchial nerve.

Also present in the visceral ganglion, especially in summer-harvested animals, was an orange auto-fluorescent pigment. This pigmented material was highly clustered in one area of the visceral ganglia and very little was found outside this area. The pigmented cells were glandular in appearance, no processes were observed. In summer animals, this pigment was in the cerebral ganglia also. Some orange fluorescing pigment was also found intracellularly within the nerve cell bodies.

Fluorescent structures in peripheral nerve

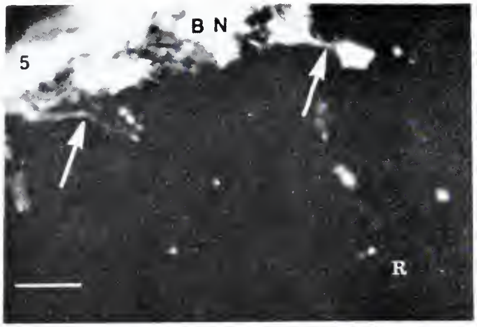
The branchial nerve contains both yellow and green fluorescing fibers as well as the yellow bead-like structures mentioned earlier. Along its length, the branchial nerve gives off many branches which run in the gill axis. One such branch could be traced in the gill axis and was seen to have further divisions leading into the individual gill filaments (Fig. 5). The branches entering the gill filament could be followed for some length, but once coming into the vicinity of the supporting rod, they would appear to merge with it. The fluorescence of these branches of the branchial nerve and elements of it that enter the gill filament appeared to be made up of green and yellow fibers. It was difficult to tell whether there was a mixing of the amines in one nerve fiber or there were two types of fibers, each emitting its own fluorescence. Essentially what was occurring was a kind of masking effect which prevented the resolution of individual fibers and colors.

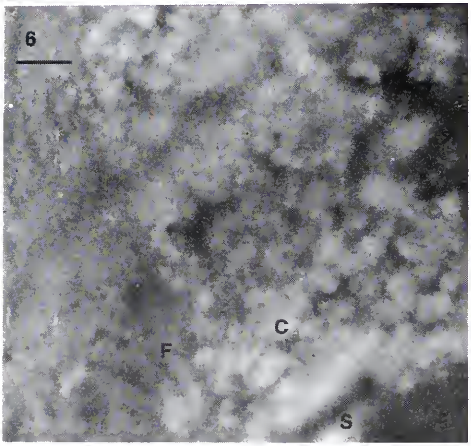
The supporting rod within the individual gill filaments exhibited specific green fluorescence which disappeared with sodium borohydride treatment. The epithelial cells in the gill filament had no specific fluorescence. Nucleated blood cells were found not only within the blood sinus but between epithelial cells of the gill filament. These blood cells exhibited a specific yellow fluorescence originating from granules within their cytoplasm and could be clearly identified as blood

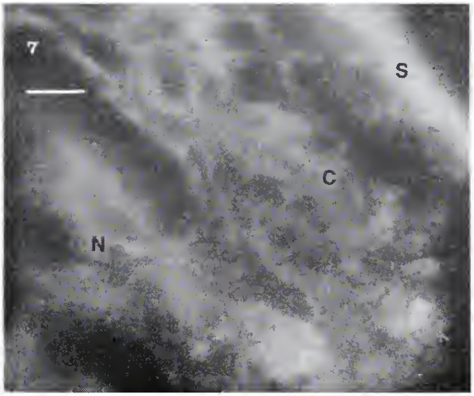
---

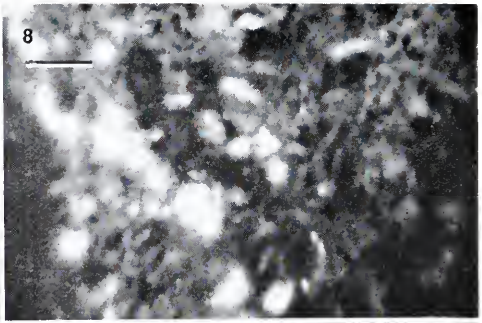
majority are green and nonfluorescent. The sheath (S) has both yellow and green fluorescing granules and fibers. Scale bar = 50 μ.

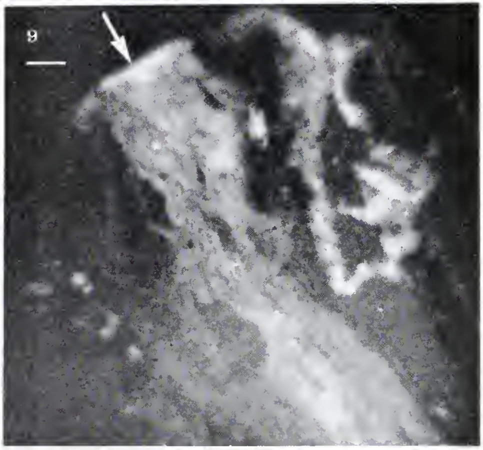
FIGURE 4. Longitudinal section through the cerebral ganglion (CG) seven days after cutting the cerebrovisceral connective (CVC). The fluorescence is intensely yellow with no detectable green. High magnification fails to reveal the discrete yellow fluorescing granules seen in the control animals; the cells now being filled with intensely fluorescent material. Scale bar = 50 μ.











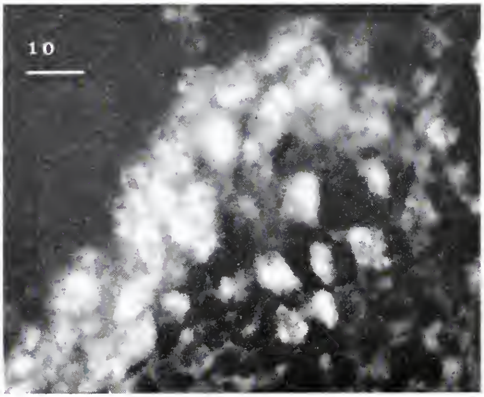


FIGURE 5. A fluorescent photomicrograph of a section through the gill axis at the origin of several filaments. A branch of the branchial nerve (BN) gives off one or more individual fibers (arrows) to each filament. Within the filament the fibers are weakly visible against the faintly green supporting rod (R). Scale bar = 50 μ.

FIGURE 6. Section of cerebral ganglion two days after the intramuscular injection of serotonin. All cells (C) and fibers (F) and the sheath (S) fluoresce yellow, indicating the relatively indiscriminate uptake of the amine. Scale bar = 20 μ.

FIGURE 7. Section of cerebral ganglion from reserpinized mussel. Despite low level of green-yellow fluorescence prolonged photographic exposure reveals an indiscriminate districells by their appearance, location, and lack of processes or attachment to any other cell.

Denervation experiments

Animals used for these experiments had their valves held open with a retractor. The cerebrovisceral connective on one side was cut about 0.5 cm from the visceral ganglion. After this operation, the animals were placed in open-ended glass tubes covered with muslin and returned to the tank for seven days.

Eight animals appeared relatively healthy at the end of the seven-day period and were prepared as described above. The cerebral ganglion was observed to have enhanced yellow fluorescence (Fig. 6). Many more cell bodies (10–11 μ in diameter) emitted the yellow fluorescence. The fluorescence intensity increased so greatly that the individual yellow fluorescing inclusions could not be distinguished. The neuropile region appeared to contain very little green fluorescence. At the beginning of the cerebrovisceral connective many more yellow fluorescing beads could be found. Also, the connective tissue sheath exhibited an enhanced yellow fluorescence.

By contrast, the visceral ganglion in general exhibited an enhanced green fluorescence. In the cortex region, this was due to enhanced green fluorescence from the nerve cell bodies. Due to the high concentration of green fibers in the neuropile, a yellow-green fluorescence was actually obtained. This yellow-green fluorescence was due to the presence of green fibers only, as demonstrated by taking thinner sections. In the neuropile region there was usually a very good supply of varicose fibers which appeared as lightened areas in the green mesh, but in the denervated animal these yellow varicose fibers were greatly reduced in number.

A close look at the transected ends of the cerebrovisceral connective also proved interesting. On the cerebral ganglion side of the transection there was an accumulation of the yellow fluorescing beads and an absence of green fluorescing fibers. On the visceral ganglion side, the connective appeared to contain enhanced green fluorescence, especially at the cut surface (Fig. 7), and no yellow beads. These results were quite dramatic when compared to the cerebrovisceral connective of control animals. This normally contains both green fibers and yellow beads distributed evenly throughout the length of the connective. The distribution

---

bution of fluorescence throughout the cortex (C), neuropile (N) and sheath (S) with no indication of discrete granules or varicosities. Scale bar = 20 μ.

FIGURE 8. Green fluorescing cells of the cerebral ganglion in an area that contained no yellow fluorescence. The fluorescence is diffuse throughout the whole cell. These cells comprise the fluorescent group in the center of the photomicrograph but many appear relatively isolated from each other. Scale bar = 20 μ.

FIGURE 9. Section through seven-day transected cerebrovisceral connective, showing the accumulation of intensely fluorescing green material on the visceral ganglion side of the cut (arrow). Scale bar = 20 μ.

FIGURE 10. A portion of the cerebral ganglion after two days exposure of whole animals to nialamide in the bathing medium. Cells and fibers show enhanced fluorescent intensity of either yellow or green, and more than the usual number of cells appear to be fluorescent. Scale bar = 20 μ.

of orange pigment with the cerebrovisceral connective seemed unaffected by the transection experiments.

The gills from these animals were also examined but did not appear to have been affected by the transection. Both green and yellow fluorescing elements were present and seemed quite normal in their appearance and distribution.

Pharmacological agents

Two animals were each injected in the posterior adductor muscle and basal portion of the foot with a total of 1 mg of serotonin in a volume of 0.2 ml. They were then placed in a beaker containing 300 ml of artificial sea water in the regular tank for temperature control and continuously aerated.

After two days they were prepared as described above. There was enhanced yellow fluorescence in both ganglia in the nerve cell bodies, in the neuropile, and in the sheath, indicating an ability to incorporate this exogenous supply of serotonin (Fig. 8). The cerebral ganglia had greater fluorescent intensity than the visceral ganglia, which appeared duller yellow. The yellow fluorescence of these cells after loading was homogenous in its intracellular appearance contrary to its normal localization in granules. In the gill axis the branches of the branchial nerve exhibited an enhanced yellow fluorescence but fibers in the individual filaments appeared normal. Where the fibers of the branchial nerve entered the gill filament, the intensity of the fluorescence was normal.

Dopamine was prepared and administered to two animals exactly as in the serotonin experiment. The animals were kept as described above for two days. The results were similar to those described for serotonin, except that the cells, nerve fibers, and sheath emitted greatly enhanced green fluorescence. In thick sections the neuropile of the visceral and cerebral ganglia gave a dull yellow fluorescence as a result of the concentration effect of excess dopamine (Falck and Owman, 1965). The gill filaments, however, tended to have a greater green distribution. The specific green fluorescence was not just confined to the supporting rod as in the normal tissue, but was also seen in the epithelial cells.

Four different animals were each injected with 2 mg of reserpine in 0.1 ml volume and kept for two days as described above. There was depletion of the monoamines as indicated by a decrease in fluorescence intensity. The exact distribution of fluorescence was obscured by a blurring effect, apparently the result of amine release from its storage sites (Fig. 9). This was observed directly and is not simply due to the long exposure time required for photomicrography of depleted tissue. It was difficult to properly discern the color green from the color yellow. The connective tissue sheath increased in fluorescence indicating that it has the ability to pick up the released amines.

The monoamine oxidase inhibitor nialamide was used to enhance intracellular pools of specific monoamine fluorescence. Two animals were bathed in an instant ocean solution that contained 1.3 mg of nialamide per ml solution for two days. In the cerebral ganglia there was an increase in the yellow and green fluorescence. The yellow vesicles in the nerve cell bodies located in the cortex increased both in number and in the intensity of fluorescence. There was also obvious increases in the number of fluorescing cells (Fig. 10). The intensity of green fluorescence of the neuropile was also enhanced.

The effect on the visceral ganglion was less pronounced except in the cortical region where the brightest green fluorescence was obtained. There was also an increase in the concentration of the non-specific orange pigment described earlier.

The gill appeared to have an enhanced but more diffuse green fluorescence. Several yellow fluorescing structures, such as blood cells and granules in the epithelium, also exhibited greater fluorescence. The green supporting rod could be seen in each gill filament but not as clearly as in the normal gill because surrounding tissue also fluoresced green.

Discussion

The foregoing description is in general agreement with those of previous authors regarding the arrangement of cell bodies into groups within the cortex and nerve fibers into tracts within the neuropile (Rawitz, 1887), the presence of distinctly green or yellow fluorescing cells and fibers (Dahl *et al.*, 1966), the predominance of green fluorescence in the visceral ganglion and of yellow fluorescence in the cerebral ganglion (Dahl *et al.*, 1966; Sweeney, 1968), and the absence of any large cells such as those found in many gastropods but not yet found in bivalves.

The specificity of yellow fluorescence from the serotonin fluorophore seems well established (Falck and Owman, 1965) and the histofluorescent localization of this amine in the nervous system and gill of *M. edulis* is in agreement with its detection there by various chemical means (Welsh and Morehead, 1960; Aiello, 1960, 1962). Identification of the green fluorescing fluorophore as that of dopamine rests on several observations. Sweeney (1963) found high concentrations of dopamine but no epinephrine or norepinephrine in the ganglia of *M. edulis* and six other bivalve species and Malanga *et al.* (1972) found an abundance of dopamine but only traces of norepinephrine in extracts of *M. edulis*. In the present experiments, treatment with formaldehyde vapor at 80° C for 3 hours instead of the usual 1 hour did not increase the extent or intensity of fluorescence even though it would have brought out fluorescence due to a secondary amine, such as epinephrine, had it been present (Falck and Owman, 1965). This kind of identification has been made for dopamine in *Anodonta cygnea* (Zs.-Nagy, 1968), *Anodonta piscinalis* (Dahl *et al.*, 1966) and *Spisula solida* (Cottrell, 1968) but not for the green fluorescence in *Sphaerium sulcatum* (Sweeney, 1968) or *Elliptio complanata* (Paparo, 1972), but it is likely that dopamine is the only catecholamine present in physiologically significant amounts in bivalve nervous systems.

The present data clearly establishes the presence of dopaminergic and serotonergic innervation of the gill of *M. edulis* in agreement with the pharmacological findings described in the introduction. Individual green and yellow fibers can be distinguished in the branchial nerve and in some of the small branches which it sends into the gill filaments as it runs anteriorly in the gill axis. Paparo (1972) reported finding only weakly green fluorescing fibers in the gills of *M. edulis* and *E. complanatus*, and Sweeney (1968) reported only yellow fluorescing fibers in the gill filaments of *S. sulcatum*. Paparo (1972) also presented clear electron micrographs of nerve fibers lying in indentations of the ciliated epithelial cells and closely apposed to the underlying supporting rod. In the present work, longitudinal sections revealed the presence of green and yellow fibers in this region but did not show the epithelial cells in the kind of orientation that allowed unequivocal identification as to type. In cross section, specifically fluorescing specks could only be seen in some sections, probably when the section contained a varicosity, and these were predominantly in the anterior portion of the filament where the ciliated cells are located but the exact point of innervation could not be determined.

Experiments with dopamine, serotonin and nialamide indicated that the supporting rod can take up dopamine and opens up the possibility that its normal green fluorescence is due to the absorption of dopamine released from adjacent nerves during preparation of the tissue. The same thing could have occurred with serotonin in Sweeney's work since the yellow fiber he describes is too large in diameter to be the fine fibers seen in electron microscopy. Although endogenous dopamine seems to be confined to the nerves and possibly the supporting rod in *M. edulis*, serotonin is found not only in the nerves but in the eosinophilic blood cells and, if nialamide is used to prevent its oxidation, in the epithelial cells of the gill. The blood cell serotonin responds to reserpine, nialamide, and exogenous serotonin as does the serotonin in the nervous system. Contrary to the report of Paparo (1972) we found no nerve cell bodies in the gill despite extensive searching, and we believe that without careful comparison of morphology, staining characteristics and relation to other cells, these serotonin-containing blood cells, which are wandering amoebocytes and sometimes send out long pseudopods, may be mistaken for nerve cell bodies.

Regarding the role of the serotonergic innervation of the gill we have no evidence for a sensory function as proposed by Sweeney (1968). In *M. edulis* the organism was never observed to respond to stimulation of the gill, we observed no sensory structures in the gill, and the nerves always appear to lie under the epithelial cells and have never been observed to pass up between them toward the surface as might be expected of sensory fibers.

A question that could not be answered from our observations was that concerning the possible presence of non-fluorescent fibers to the gill. There are many non-fluorescent fibers present in the origin of the branchial nerve. Bouffard (1970) showed sections in which all the fibers of the branchial nerve were histochemically positive for acetylcholine esterase. In almost identically appearing sections through the branchial nerve as it innervates individual filaments, we found that all the fibers are histochemically fluorescent. Because of their close packing and very small diameter one must concede the possible presence of non-fluorescent fibers in each small group and these were looked for in the present study by phase contrast microscopy but without success. In view of our present finding that exogenous amines can be taken up by nerves not exhibiting specific fluorescence for their endogenous presence it is possible that all fibers become fluorescent during preparation. The same could apply to the deposition of the cobalt sulfide precipitate in the cholinesterase staining and the problem remains unresolved.

The branchial nerve comes from the visceral ganglion and the present study indicates that dopaminergic and serotonergic cell bodies in that ganglion send fibers into the branchial nerve. The serotonin-containing cells are larger but fewer in number, and this is in keeping with the predominance of green color in the branchial nerve. Transection of the cerebrovisceral connective decreased the amount of yellow fluorescing granules and varicose fibers in the connective on the visceral ganglion side of the cut, in the neuropile and in the cortex but did not alter the intensity of fluorescence in cell bodies or fibers in the branchial nerve. This indicates that fibers from the cerebral ganglion do not simply pass through the visceral ganglion on their way to the gill but terminate there, apparently on several kinds of cells. Transection of the connective caused an increase in green fluorescence in the ganglion but did not affect the branchial nerve. On the cerebral ganglion side of the cut and in the cerebral ganglion itself, there was a great enhancement of yellow fluorescence. The role of this cerebral-to-visceral-ganglion transport of serotonin has not yet been determined but some preliminary experiments (Catapane, Aiello and Stefano, 1974) suggest that it has to do with stimulating the cilioexcitatory serotonergic fibers originating in the visceral ganglion. Dahl *et al.* (1966) performed a similar experiment in *A. piscinalis* with similar results regarding the increase in serotonin in the cerebral ganglion, cortex and neuropile and its decrease in the visceral neuropile. They did not observe yellow fluorescing cells in the visceral ganglion cortex and since there is no information on the function of the branchial nerve in that species we can not assess the physiological significance of their experiment.

An observation of general interest which we made consistently throughout this study is that in *M. edulis* endogenous, intracellular yellow fluorescence is always in granules whereas green fluorescence always appears to be homogenously distributed. This granular localization of serotonin may be lost following treatment with reserpine, nialamide or exogenous serotonin, suggesting that it is its normal, physiological condition to be so localized. Zs.-Nagy (1968) identified the site of dopamine localization in the cerebral ganglion of *A. cygnea* to be 1000 Angstrom diameter dense core vesicles, which would be below the limit of resolution of the light microscope. He did not comment on the serotonin sites. Dahl *et al.* (1966) mention yellow fluorescing granules but do not comment on the green fluorescing cells. In the absence of specific statements by other authors we do not know how general a condition this is in bivalves but it appears to be consistent in *M. edulis*.

The significance of the orange auto-fluorescing pigment observed by ourselves and others in both monoaminergic and other neurones remains to be clarified (Zs.-Nagy, 1968; Dahl *et al.*, 1966) but we can be sure that in *M. edulis* it is subject to seasonal change. The fact that this pigment is increased by nialamide treatment and that prolonged exposure to UV light slowly converts it to a yellow fluorescing pigment suggests that it might be metabolically related to serotonin. The increase in the number of specific yellow-fluorescing cells following treatment with nialamide might be explained if one assumed that cells which originally contained orange autofluorescent pigment were potentially serotonergic and accumulated serotonin when its oxidation was inhibited by nialamide.

SUMMARY

Monoamine localization was accomplished in *Mytilus edulis* by the use of histofluorescence. Intracellular stores of dopamine and serotonin were found to be

synthesized in the proper neuron and transported down the axon to the terminal varicosities.

Most of the cells in the cortex of the cerebral and visceral ganglia were non-fluorescent. Of the fluorescent cells, serotonin predominated in the cerebral ganglion and dopamine predominated in the visceral ganglion. There was a net flow of serotonin in the cerebro-visceral connective from the cerebral to the visceral ganglion and a net flow of dopamine in the opposite direction.

Serotonin fluorescence was localized in intracellular granules in neurons and blood cells. Dopamine fluorescence was distributed homogenously in neurons and in the supporting rod of the gill. The visceral ganglion supplies the gill with nerve fibers of both types.

Exogenously supplied serotonin and dopamine were taken up by both kinds of nerve cells and by some other tissues. Endogenous stores of both amines were altered in content and distribution by reserpine and by nialamide.

The distribution of monoamine in the nervous system and gill lends further support to the notion of a dual innervation mechanism controlling ciliary activity in the gill.

LITERATURE CITED

Aiello, E., 1960. Factors affecting ciliary activity on the gill of the mussel *Mytilus edulis*. *Physiol. Zool.*, **33**: 120–135.

Aiello, E., 1962. Identification of the cilioexcitatory substance present in the gill of the mussel *Mytilus edulis*. *J. Cell. Comp. Physiol.*, **60**: 17–21.

Aiello, E., 1965. The fate of serotonin in the cell of the mussel *Mytilus edulis*. *Comp. Biochem. Physiol.*, **14**: 71–82.

Aiello, E., 1970. Nervous and chemical stimulation of gill cilia in bivalve molluscs. *Physiol. Zool.*, **43**: 60–70.

Aiello, E., and G. Guideri, 1964. Nervous control of ciliary activity. *Science*, **146**: 1692–1693.

Aiello, E., and G. Guideri, 1965. Distribution and function of the branchial nerve in the mussel. *Biol. Bull.*, **129**: 431–438.

Aiello, E., and G. Guideri, 1966. Relationship between 5-hydroxytryptamine and nerve stimulation of ciliary activity. *J. Pharmacol. Exp. Therap.*, **154**: 517–523.

Aiello, E., and A. Paparo, 1974. A role for acetylcholine in the regulation of ciliary activity. *Comp. Gen. Pharmacol.*, in press.

Blaschko, H., and A. Milton, 1960. Oxidation of 5-hydroxytryptamine and related compounds by *Mytilus* gill plates. *Brit. J. Pharmacol.*, **15**: 42–46.

Bouffard, T. G., 1970. Functional histology and histochemistry of the gill and nervous system of the mussel, *Mytilus edulis* *Ph.D. thesis, Fordham University*, New York, 284 pp.

Bulbring, E., J. H. Burn and H. J. Shelly, 1953. Acetylcholine and ciliary movement in the gill plates of *Mytilus edulis*. *Proc. Roy. Soc. London Series B.*, **141**: 445–466.

Catapane, E., E. Aiello and G. Stefano, 1974. Ganglionic mediation mechanisms of lateral cilia in *Mytilus edulis* gill. *The Physiologist*, **17**: 372.

Corrodi, H., N. Hillarp and G. Jonsson, 1964. Fluorescence methods for the histochemical demonstration of monoamines. 3. Sodium borohydride reduction of the fluorescent compounds as a specificity test. *J. Histochem. Cytochem.*, **12**: 582–586.

Cottrell, G. A., 1968. Amines in molluscan nervous tissue and their subcellular localization. Pages 353–364 in J. Salanki, Ed., *Invertebrate Neurobiology*. Plenum Press, New York.

Dahl, E., B. Falck, M. Lindquist and C. Von Mecklenburg, 1962. Monoamines in mollusc neurons. *Kungliga Fysiografiska Sallskapets I Lund Fordhandlingar,* **32**: 89–92.

Dahl, E., B. Falck, C. Von Mecklenburg, H. Myhrberg and E. Rosengren, 1966. Neuronal localization of dopamine and 5-hydroxytryptamine in some molluscs. *Z. Zellforsch. Mikrosk, Anat.,* **71**: 489–498.

Falck, B., N. Hillarp, G. Thieme and A. Torp, 1962. Fluorescence of catecholamines and related compounds condensed with formaldehyde. *J. Histochem. Cytochem.,* **10**: 348–354.

Falck, B., and C. Owman, 1965. A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund., Section 2,* No. **7**: 5–23.

Favorsky, B. A., 1930. Eine Modifikation des Silber Impregmationsverfahrens Ramon Y Cajal für das periphere Nervensystem. *Anatomischer Anzeiger,* **70**: 376–378.

Field, I. A., 1922. Biology and economic value of the sea mussel, *Mytilus edulis. Bull. U.S. Fish. Bur.,* **38**: 127–257.

Gosselin, R. E., K. E. Moore and A. S. Milton, 1962. Physiological control of molluscan gill cilia by 5-hydroxytryptamine. *J. Gen. Physiol.,* **46**: 277–296.

Grave, C., and F. O. Schmitt, 1925. A mechanism for the coordination and regulation of ciliary movement as revealed by microdissection and cytological studies of ciliated cells of molluscs. *J. Morphol.,* **40**: 479–516.

Koshtoyants, K. S., G. A. Buznikov and B. N. Manukhin, 1961. The possible role of 5-hydroxytryptamine in the motor activity of embryos of some marine gastropods. *Comp. Biochem. Physiol.,* **3**: 20–26.

Malanga, C. J., G. R. Wenger and E. Aiello, 1972. Endogenous dopamine in bivalve gills. *Comp. Biochem. Physiol.,* **43A**: 825–830.

Marsden, C. A., and G. A. Kerkut, 1969. The cellular localization of monoamines in invertebrates using the Edwards-Pearse Tissue Freeze Dryer. Pages 327–360 in G. A. Kerkut, Ed., *Laboratory Experiments in Physiology and Biochemistry,* Vol. 2. Academic Press, New York.

Paparo, A., 1972. Innervation of the lateral cilia in the mussel *Mytilus edulis. Biol. Bull.,* **143**: 592–605.

Paparo, A., and E. Aiello, 1970. Cilio-inhibitory effects of branchial nerve stimulation in the mussel *Mytilus edulis. Comp. Gen. Pharmacol.,* **1**: 241–250.

Rawitz, B., 1887. Das Zentrale Nervensystem der Acephalen. *Jenaische Zeitschrift fur Medizin und Naturwissenschaft.,* **20**: 384–460.

Schor, S. K., 1965. Serotonin and adenosine triphosphate: synergistic effect on beat frequency of cilia of mussel gill. *Science,* **148**: 500–501.

Setna, S. B., 1930. The neuro-muscular mechanism of the gill of *Pecten. Quart. J. Microscop. Sci.,* **72**: 365–391.

Splittstosser, P., 1913. Zur morphologie des Nervensystems von *Anodonta cellensis* Schrot. *Z. Wiss. Zool.,* **104**: 388–470.

Stefano, G. B., and E. Aiello, 1972. Localization of monoamines in the cerebral and visceral ganglia of *Mytilus edulis. Amer. Zool.,* **12**: xxxvi (abstract 413).

Sweeney, D., 1963. Dopamine: Its occurrence in molluscan ganglia. *Science,* **139**: 1051.

Sweeney, D., 1968. The anatomical distribution of monoamines in a fresh water bivalve mollusc, *Sphaerium sulcatum* (L.). *Comp. Biochem. Physiol.,* **25**: 601–614.

Takahashi, K., and A. Murakami, 1968. Nervous inhibition of ciliary motion in the gill of the mussel, *Mytilus edulis. J. Fac. Sci. Univ. Tokyo Sect. IV,* **11**: 359–372.

Von Euler, U. S., 1961. Occurrence of catecholamines in Acrania and invertebrates. *Nature,* **190**: 170–171.

Welsh, J. H., 1970. Phylogenetic aspects of the distribution of biogenic amines. Pages 75–94 in J. J. Blum, Ed., *Biogenic Amines as Physiologic Regulators.* Prentice-Hall, Englewood Cliffs, New Jersey.

WELSH, J. H., 1972. Catecholamines in the invertebrates. *Handbook Exp. Pharmacol.,* **33**: 79–109.

WELSH, J. H., AND M. MOORHEAD, 1960. The quantitative distribution of 5-hydroxytryptamine in the invertebrates, especially in their nervous systems. *J. Neurochem.,* **6**: 146–169.

ZS.-NAGY, I., 1968. Histochemical and electron-microscopic studies on the relation between dopamine and dense-core vesicles in the neurons of *Anodonta cygnea* L. Pages 69–84 in J. Salanki, Ed., *Neurobiology of Invertebrates.* Plenum Press, New York.

Reference: *Biol. Bull.*, **148**: 157–164. (February, 1975)

OXYGEN CONSUMPTION IN ECHINODERMS FROM SEVERAL GEOGRAPHICAL LOCATIONS, WITH PARTICULAR REFERENCE TO THE ECHINOIDEA[1]

STEVEN K. WEBSTER

Department of Biological Sciences, California State University,
San Jose, California 95192

Little information is available on respiration of echinoderms (Bliss and Skinner, 1963; Prosser and Brown, 1962). This study presents data obtained for the five classes of living echinoderms from several geographical locations, including the Monterey Peninsula, California; Punta Banda and Puertecitos, Baja California, Mexico; Savai'i, Western Samoa; St. John, U. S. Virgin Islands; and Dunedin, New Zealand. Articles by Farmanfarmaian (1966), Moore (1966), and Lewis (1967), point out the general lack of information concerning whole body respiration (oxygen consumption) in all but a few echinoids. Concurrent with an investigation of the respiratory physiology of the purple sea urchin, *Strongylocentrotus purpuratus,* in relation to reproduction, and while engaged in brief excursions to Western Samoa and to the U. S. Virgin Islands, the author was able to obtain whole body oxygen consumption data for several tropical and temperate echinoderms. In addition, laboratory studies were conducted to compare the respiratory physiology of the Pacific Coast deep water echinoid, *Allocentrotus fragilis*, and the purple sea urchin, *S. purpuratus*. This comparison provides additional information concerning the relative contributions of specific body components to whole body respiration in echinoids.

MATERIALS AND METHODS

The investigations on location were conducted with freshly collected animals according to the methods described in Webster and Giese, 1974. Closed chamber respirometry was conducted with the YSI oxygen electrode at the temperatures at which the animals were collected. All oxygen depletion experiments were of two hours' duration, and readings were taken at half-hour intervals. Large animals were tested singly in the respirometer, and small individuals were used in groups of three to six, and their weights were averaged for the calculation of the Q_{O_2}.

Laboratory investigations were conducted with the Beckman oxygen electrode apparatus described in Webster and Giese, 1974. All data presented represent a minimum of three experiments with at least three individuals of each species. Where a wide size range was investigated, the extremes are indicated. In most cases animals of similar size within a species were used, and average wet weights are indicated. Animals tested in the laboratory were held without food in aquaria for at least one week prior to experimentation.

---

[1] Supported by a National Science Foundation Graduate Fellowship to the author.

Laboratory investigations of *Allocentrotus fragilis* were conducted according to the methods described for *S. purpuratus*. The Beckman polarographic macro-oxygen electrode was used for determinations of whole body oxygen consumption and was coupled to a potentiometric strip chart recorder to produce a continuous print-out of the oxygen depletion curve. The Instrumentation Laboratories, Inc. #17365 needle oxygen electrode was used for determinations of perivisceral fluid oxygen partial pressure (pvfO_2), and was coupled to the chart recorder.

Collections of *A. fragilis* were made in April, 1971, and in February, 1972, with a beam trawl from the R. V. Proteus of Stanford University. These collections were made at a depth of about 150 meters at 36° 41′ N., 122° 04′ W., off the Monterey Peninsula, California. Specimens of *A. fragilis* were held in dark aquaria at 6° C for several days prior to use in these investigations.

Data for *Evechinus chloroticus* and *Goniocidaris umbraculum* were obtained at Dunedin, New Zealand by William Johnson of the Hopkins Marine Station. Animals were held in the laboratory at 13° C for several days prior to use in the investigation.

Eupentacta quinquesemita was collected from among tubes of chaetopterid polychaetes of wharf pilings in Monterey Harbor. *Parastichopus californicus*

TABLE I

Q_{O_2} of echinoderms from several geographical locations

Class, species	Q_{O_2} (µl/g/hr)	Wet weight (g)	Temp. (C)	Location
Asteroidea				
Dermasterias imbricata	7.5	23.5	13	Monterey, California
Leptasterias aequalis	26.8	1–2	13	Monterey, California
Mediaster aequalis	15.3	15.3	13	Monterey, California
Orthasterias columbians	25.4	20.8	13	Monterey, California
Patiria miniata	10.5	11.6	13	Monterey, California
Pisaster brevispinus	12.3	19.9	13	Monterey, California
Pisaster giganteus	25.4	1.3	13	Monterey, California
Pycnopodia helianthoides	9.8	26.2	13	Monterey, California
Zoroaster evermanni	5.6	52.9	8	Monterey, California
*Astropecten aramtus**	17.4	27.1	16	Punta Banda, B. C., Mexico
*Heliaster kubiniji**	17.8	106.2	16	Puertecitos, B. C., Mexico
*Linkia laevigata**	2.2	100.3	23	Savai'i, Western Samoa
*Coscinasteria calamaria***	9.8	33–81	13	Dunedin, New Zealand
*Pentagonaster pulchellus***	3.8	12.0	13	Dunedin, New Zealand
Ophiuroidea				
Amphiodia occidentalis	3.0	0.5–1.0	13	Pacifica, California
Holothuroidea				
Eupentacta quinquesemita	17.5	5.5	13	Monterey, California
Leptosynapta inhaerens	13.9	2.4	13	Monterey, California
Parastichopus californicus	19.2	27.3	13	Monterey, California
Crinoidea				
Florometra serratissima	26.7	4.5	8	Monterey, California

* Data obtained in the field with freshly collected animals and the YSI oxygen electrode.

** Data obtained in the laboratory with the YSI electrode. Remainder of the data obtained in the laboratory with the Beckman oxygen electrode.

was collected from granite boulders under a kelp bed at Carmel, California. The *Leptosynapta inhaerens* is truly infaunal, and specimens were collected from damp sand during a low tide at Pacifica, California.

The *Amphiodia occidentalis* was collected from nearly anaerobic black sand under rocks at Pedro Point in Pacifica, California.

Florometra serratissima was collected by beam trawl at a depth of about 150 meters from the R. V. Proteus of Stanford University.

Experiments and Results

Oxygen consumption rates for the Asteroidea, Ophiuroidea, Holothuroidea, and Crinoidea are presented in Table I. The data for the Echinoidea are presented in Table II.

Discussion

The low Q_{O_2} exhibited by *Leptosynapta inhaerens* (Table I) may indicate a heavy reliance upon anaerobic respiration, particularly during low tides when water circulation under the rocks is minimal. In addition to the lower Q_{O_2}, other adaptions such as vermiformity and the loss of some internal complexity are demonstrated by the infaunal *L. inhaerens* as compared with the two epifaunal species. These relationships were found among infaunal as compared with epifaunal anemones by Sassaman and Mangum (1972). Lewis (1967) found the same to be true in tropical echinoids.

The relatively high Q_{O_2} of the crinoid, *Florometra serratissima,* may be due to its high level of activity. When disturbed, this animal exhibits active swimming behavior. Some activity persisted in the respirometer during the oxygen depletion experiments, and may be responsible for the relatively high Q_{O_2} recorded. The relatively high index of living material in this species may also be a factor, although determinations of body component indices were not made.

Table II

Q_{O_2} *of echinoids from several geographical locations*

Species	Q_{O_2} (μl/g/hr)	Wet weight (g)	Temp. (C)	Location
Echinoidea				
*Allocentrotus fragilis**	5.17	50.4	8	Monterey, California
*Strongylocentrorus purpuratus**	8.00	55.0	13	Pigeon Point, California
*Strongylocentrorus franciscanus**	6.72	44.7	13	Pigeon Point, California
Lytechinus anamesus	14.90	3.7	16	Punta Banda, B. C., Mexico
Echinometra mathaei	4.70	28.0	23	Savai'i, Western Samoa
Echinometra lucunter	13.30	3.1	28	St. John, U. S. Virgin Is.
Tripneustes esculentus	15.60	5.2	28	St. John, U. S. Virgin Is.
*Evechinus chloroticus***	5.41	760.0	13	Dunedin, New Zealand
*Goniocidaris umbraculum***	4.00	12.1	13	Dunedin, New Zealand

* Data obtained with Beckman oxygen electrode in the laboratory.

** Data obtained in the laboratory with YSI oxygen electrode by William Johnson, Hopkins Marine Station. Remainder of the data obtained in the field with YSI oxygen electrode. Method described in text. (Webster and Giese, 1974.)

Reference to Table I indicates that in the asteroids, as in the echinoids, the highest oxygen consumption rates appear among the smallest individuals, regardless of the environmental temperatures, a fact well in keeping with information on all types of animals (Prosser and Brown, 1962). In fact, the lowest Q_{O_2} is found in *Linkia laevigata* at 23° C, the warmest temperature of any of the areas studies. The relatively large size of these individuals may be of greater importance than the ambient temperature in determining the Q_{O_2} of the animal. Of the species of asteroids investigated at Monterey, California (13° C), the smallest individuals exhibit the highest Q_{O_2}. It must be noted, however, that other factors which may have an effect on oxygen consumption have not been considered. These include activity, nutritional condition, and differences in microhabitat.

Among the echinoids (Table II), with the exception of *Echinometra mathaei*, the warm-water species exhibit a uniformly higher Q_{O_2} than their cold-water counterparts. All oxygen consumption rates are within the same order of magnitude found for *S. purpuratus* (Webster and Giese, 1974). It should be noted that the highest oxygen consumption rates are found among the smaller sea urchins tested, a fact which must be considered in addition to the warmer environmental temperatures of these species, in interpreting these data.

The comparatively low Q_{O_2} of *E. mathaei* is of interest as it is lower (at 28° C) than that of *S. purpuratus* (at 13° C) and *A. fragilis* (at 8° C). In light of the warmer environmental temperature and smaller size of the *E. mathaei*, it would be expected to exhibit a higher Q_{O_2} (Lewis, 1967).

The comparison of *S. purpuratus* and *A. fragilis* (Table III) reflects basic differences in the body components of these species which may account for the observed differences between them in respiratory physiology. The comparison of body wall indices shows that of *A. fragilis* to be less than half that of *S. purpuratus* (25.16 and 55.00, respectively). Because 90% or more of the whole body Q_{O_2} is attributable to the body wall component in *S. purpuratus*, little oxygen is left for transport to the perivisceral coelom and internal tissues (Webster and Giese, 1974), resulting in the low perivisceral fluid oxygen partial pressure ($PvfO_2$) of 45 mm Hg. The $PvfO_2$ of *A. fragilis* at 115 mm Hg is much higher, and can be attributed to the low body wall index which leaves substantially more oxygen for transport to the internal tissues. Body wall oxygen consumption in both species is from two to three times the whole body Q_{O_2} (Table III).

In his study of *Evechinus chloroticus*, Johnson found that the body wall Q_{O_2} was lower than that for the whole animal (3.04 μl/g/hr for the body wall, 5.41 μl/g/hr for the whole animal). This is contrary to the results for *S. purpuratus* and *A. fragilis*, and may have its explanation in the very large size and high gonad

TABLE III

A comparison of S. purpuratus *with* A. fragilis: *factors of significance to the respiratory physiology of echinoids*

Species	Q_{O_2} (μl/g/hr)	Body Wall Index	$PvfO_2$ mm Hg	Body Wall Q_{O_2} (μl/g/hr)	Temp. (C)
A. fragilis	5.17	25.16	115	12.7	8
S. purpuratus	8.00	55.00	45	22.0	13

Table IV

The gonad index and whole body Q_{O_2} for five Allocentrotus fragilis *at 8° C*

Gonad index	Whole body Q_{O_2} (μl/g/hr)
2.75	6.98
2.43	5.05
4.00	4.06
5.09	8.62
7.42	7.08

index of the *Evechinus chloroticus* tested. Additional data will be required for the resolution of this question.

Although the conclusion depends upon additional supporting data, it appears that there is no relationship between whole body oxygen consumption and reproductive condition (gonad index) in *Allocentrotus fragilis* (Table IV). These data are in agreement with those concerning whole body Q_{O_2} and the gonad index in *S. purpuratus* (Webster and Giese, 1974). Unfortunately, collections were not possible during the peak of the reproductive cycle in *A. fragilis* (November and December) because of storms, and tests were not made at this critical part of the reproductive cycle. However, between the highest (7.42) and lowest (2.43) periods of the gonad index recorded, no significant differences in perivisceral fluid ppO_2 are found. The $PvfO_2$ is 115–120 mm Hg regardless of the gonad index within the range tested. $PvfO_2$ is less variable in *A. fragilis* than in *S. purpuratus.*

Lewis (1967) found higher rates among tropical, as compared with temperate echinoids. Farmanfarmaian (1966) reports higher Q_{O_2} for *Arbacia* and *Strongylocentrotus* at 20° C than for *Echinocardium* at 15° C. Belman and Giese (1974) report a Q_{O_2} for an antarctic echinoid, *Sterechinus neumayeri*, comparable to that of temperate echinoids, and lower than that of most tropical echinoids. As stated by Kinne (1964), however, it must be recognized that an animal reacts to its total environment rather than to a single entity such as temperature, and the combined effects of two or more components of the environment must often be considered. Body size, nutritional condition, activity, and microhabitat may all be factors in determining the Q_{O_2} of any particular echinoid (Farmanfarmaian, 1966). The Q_{O_2} of epifaunal forms is higher than that of their infaunal counterparts. That this is true for anemones is demonstrated by Sassaman and Mangum (1972) and for tropical echinoids by Lewis (1967).

The prediction that tropical echinoids have a higher Q_{O_2} than temperate species holds true for all those tested except *Echinometra mathaei* from Western Samoa (Table II). Rather than reflecting higher rates under warmer environmental conditions, these data may reflect the generally smaller body size of the tropical echinoids in this study. The comparatively low Q_{O_2} exhibited by *E. mathaei* defies explanation, although the average weight of 28.0 g is much larger than the average weights of the other tropical species studied. The *E. mathaei,* with a Q_{O_2} of 4.70 μl/g/hr at 23° C, are respiring at about half the rate of *S. purpuratus* of similar size at 13° C.

TABLE V

Oxygen consumption of representative aquatic species from several phyla

Phylum, species	Q_{O_2} (μl/g/hr)	Temp. (C)	Reference
Porifera			
Microciona prolifera	115	37	Bliss and Skinner, 1963
Cnidaria			
Astrangia danae	26		Sassaman and Mangum, 1972
Haloclava producta	30		Sassaman and Mangum, 1972
Metridium senile	100		Sassaman and Mangum, 1972
Annelida			
Arctonoe vittata	27–167	13	Webster, unpublished
Diopatra cuprea	50		Mangum, Kushins and Sassaman, 1970
Mollusca			
Cryptochiton stelleri	6	13	Webster, unpublished
Helix pomatia	45–3070	28	Kerkut and Laverack, 1956
Arthropoda (Crustacea)			
Cambarus clarkii	200	25	Maloeuf, 1937
Cirolana harfordi	95–463	13	Webster, unpublished
Gnathophausia ingens	48	4.5	Childress, 1971
Spirontocaris securifrons	77	6.5	Fox, 1936
Opisthopus transversus	11–153	13	Webster, unpublished
Echinodermata			
Antedon petasus	24	8	Fox, 1936
Arbacia punctulata	16	20	Boolootian and Cantor, 1965
Asterias forbesii	100	25	Maloeuf, 1937
Holothuria forskali	2.7	10	Fox, 1936
Ophiura terturata	11	10	Fox, 1936
Ophiura terturata	21	17	Fox, 1936
Pteraster tesselatus	10.5	10–12	Johansen and Peterson, 1971
Chordata (Vertebrata)			
Ambystoma maculatum	120	20	Whitford and Hutchinson, 1967

In comparing the oxygen consumption data for all of the tropical species, it is evident that they have oxygen consumption rates that are similar to those of temperate (*S. purpuratus* and *S. franciscanus*) and Antarctic (*Sterechinus neumayeri*) echinoids of equivalent size, suggesting their adaptation to higher temperature. Certainly this reflects some form of thermal adaptation (see Hochachka and Somero, 1973) among tropical, temperate, and polar echinoderms, but the biochemical basis of this has yet to be determined.

In his table of comparative oxygen consumption rates among representatives of several major phyla, Nicol (1967) cites data in which the echinoderms exhibit similarly low rates in comparison to the other groups. I believe the relatively low Q_{O_2} exhibited by the echinoderms as a group is based on three factors. This, of course, assumes that other factors which might affect oxygen consumption (nutrition, activity, reproductive condition, *etc.*) are equal, an assumption which is questionable at best.

The first factor is the relatively inefficient transport of oxygen to the internal tissues of echinoderms. This inefficiency is probably most pronounced among the echinoids, and least important among the holothuroids with respiratory trees and respiratory pigments. Among the phyla listed in Table V, either a circulatory system including respiratory pigments supplies the internal tissues, or ambient sea water may be circulated as in the Anthozoa, past both sides of a relatively thin body wall; or, in the case of the sponges, ambient water is circulated in close proximity to all living cells. Thus, it appears that among these groups (Table V) the echinoderms exhibit the lowest level of efficiency in providing the internal tissues and body fluids with oxygen.

A second factor is the relatively high proportion of metabolically inert skeletal material in some echinoderms (the ophiuroids, in particular) and the large volume of perivisceral fluid, with its very low metabolic activity, in others (echinoids and holothuroids). Thus, as has been shown for the echinoids (Webster and Giese, 1975), only a small component of the body (the body wall tissue) accounts for 90% of the oxygen consumption of the animal.

A third possible factor resulting in the relatively low Q_{O_2} among echinoderms is their low level of activity when compared with many other phyla. It is difficult to differentiate standard from active metabolic rates among the echinoids. The authors cited in Table V, however, often point out the differences in standard and active metabolism, resulting in the highly variable Q_{O_2} data given for some species.

SUMMARY

1. Whole body oxygen consumption data are determined for a variety of echinoid, asteroid and holothuroid species, and for one ophiuroid and one crinoid.

2. Comparisons of tropical and temperate asteroids indicate no correlation of oxygen consumption rate with environmental temperature among the individuals tested. As is the case for echinoids, body size appears to be an important factor in determining the Q_{O_2} of a particular species.

3. Whole body oxygen consumption data are determined for several temperate and tropical echinoid species, and for a deep-water echinoid of the Pacific Coast.

4. A comparison of respiratory physiology in *S. purpuratus* and *A. fragilis* is made, with particular attention to the body wall and gonad indices.

5. Comparisons of tropical and temperate echinoid oxygen consumption rates reveal that, when animals of similar size are compared, the Q_{O_2} of the tropical forms is similar to that of their temperate counterparts.

6. The generally smaller body size of the tropical species tested results in the higher oxygen consumption rates which they exhibit as compared with temperate echinoids.

LITERATURE CITED

BELMAN, B., AND A. GIESE, 1974. Oxygen consumption of an asteroid and an echinoid from the antarctic. *Biol. Bull.*, **146**: 157–164.

BLISS, D., AND D. SKINNER, 1963. *Tissue Respiration in Invertebrates.* American Museum of Natural History, New York, 138 pp.

BOOLOOTIAN, R. A., AND M. H. CANTOR, 1965. A preliminary report on respiration, nutrition, and behavior of *Arbacia punctulata. Life Sci.*, **4**: 1567–1571.

CHILDRESS, J., 1971. Respiratory adaptations to the oxygen minimum layer in the bathypelagic mysid *Gnathophausia ingens. Biol. Bull.*, **141**: 109–112.

Farmanfarmaian, A. A., 1966. The respiratory physiology of echinoderms. Pages 245–265 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience Publishers, New York.

Fox, H. M., 1936. The activity and metabolism of poikilothermic animals at different latitudes. *Proc. Zool. Soc. London,* **A109**: 141–156.

Hochachka, P. W., and G. N. Somero, 1973. *Strategies of Biochemical Adaptation.* W. B. Saunders Co., Philadelphia, 358 pp.

Johansen, K., and J. A. Peterson, 1971. Gas exchange and active ventilation in a starfish, *Pteroster tessalatus. Z. Vergl. Physiol.,* **71**: 365–381.

Kerkut, G. A., and M. S. Laverack, 1956. The respiration of *Helix pomatia,* a balance sheet. *Exp. Biol.,* **34**: 97–105.

Kinne, O., 1964. Non-genetic adaptation to temperature and salinity. *Helgolaender Wiss. Meeresunters,* **9**: 433–458.

Lewis, J. B., 1967. Comparative respiration of tropical echinoids. *Comp. Biochem. Physiol.,* **24**: 649–652.

Maloeuf, N. S. R., 1937. Studies of the respiration (and the osmoregulation) of animals. I. Aquatic animals without an oxygen transporter in their internal medium. *Z. Vergl. Physiol.,* **25**: 1–28.

Mangum, C. P., L. J. Kushins and C. Sassaman, 1970. Responses of intertidal invertebrates to low oxygen conditions. *Amer. Zool.,* **10**: 516–517.

Moore, H. B., 1966. Ecology of echinoids. Pages 73–85 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience Publishers, New York.

Nicol, J. A., 1967. *The Biology of Marine Animals.* Sir Isaac Pitman Sons, Ltd., London.

Prosser, C. L., and F. Brown, 1962. *Comparative Animal Physiology.* W. B. Saunders, Philadelphia, 688 pp.

Sassaman, C., and C. P. Mangum, 1972. Adaptations to environmental oxygen levels in infaunal and epifaunal anemones. *Biol. Bull.,* **143**: 657–678.

Webster, S. K., and A. C. Giese, 1975. Oxygen consumption of the purple sea urchin with special reference to the reproductive cycle. *Biol. Bull.,* **148**: 165–180.

Whitford, W. G., and V. H. Hutchinson, 1967. Body size and metabolic rate in salamanders. *Physiol. Zool.,* **40**: 127–133.

Reference: *Biol. Bull.*, **148**: 165–180. (February, 1975)

OXYGEN CONSUMPTION OF THE PURPLE SEA URCHIN WITH SPECIAL REFERENCE TO THE REPRODUCTIVE CYCLE[1]

STEVEN K. WEBSTER AND ARTHUR C. GIESE

Department of Biological Sciences, California State University, San Jose, California 95192
and Department of Biological Sciences, Stanford University, Stanford, California 94305

Steen (1965) reported that the rate of oxygen consumption per unit weight (Q_{O_2}) of intact sea urchins is about one tenth the theoretical value calculated from summed tissue respiration and attributed this discrepancy to inefficiencies in the movement of external sea water and internal respiratory media (ambulacral and perivisceral fluids) over respiratory epithelia.

Giese, Farmanfarmaian, Hilden and Doezema (1966) reported that the Q_{O_2} per unit weight remained the same throughout the annual reproductive cycle of the purple sea urchin (*Strongylocentrotus purpuratus*) at the peak of which the organic content of the body approximately doubles. The increase in organic material in the body is not taken into account by a Q_{O_2} so measured because the specific gravity of the organic matter is very similar to that of the body fluid displaced as the gonads grow; the total volume of a sea urchin remains much the same during the reproductive cycle except for growth. To relate the oxygen consumption of the sea urchin to its organic content the Q_{O_2} was determined per unit nitrogen. On this basis the Q_{O_2} declined during growth of the gonads, minimal values' being found at the peak of the reproductive cycle when the gonads were of maximal size. The authors postulated that the oxygen consumption of the intact sea urchin is limited by the respiratory surface and by inefficient convective oxygen transport from ambient sea water to internal tissues. When the gonads reach maximal size the supply of oxygen to the tissues is poorest. As indirect evidence for this contention they point out that the sum of oxygen consumption of the individual body components measured in a dissected sea urchin is always greater than that for the intact organism.

Johansen and Vadas (1967) studied oxygen consumption in relation to ambient and perivisceral fluid oxygen partial pressure (ppO_2) in three sea urchins of the genus *Strongylocentrotus: S. purpuratus, S. franciscanus* and *S. drobachiensis.* They concluded that the Q_{O_2} measured directly is more closely related to internal than external ppO_2. They did not, however, study the relation between the reproductive state and oxygen consumption.

The present study aims to elucidate, by polarographic oxygen electrode measurements of the perivisceral fluid oxygen partial pressure ($PvfO_2$) and the ambient sea water oxygen partial pressure (AO_2), the apparent paradoxical decline in Q_{O_2} per unit nitrogen with increase in organic matter of the purple sea urchin during the course of the reproductive cycle. Also considered are the effects of

---

[1] Supported by research grant 2-FAA-504 by the National Science Foundation to Arthur C. Giese, and by a National Science Foundation Graduate Fellowship to Steven K. Webster.

body size, air exposure, and the possible relative contribution of the body wall to the total oxygen consumption of an intact sea urchin.

MATERIALS AND METHODS

Collecting sites

The sea urchins were collected monthly or bi-monthly on the San Mateo County coast, California (37°20′ N. Latitude) from populations in similar low intertidal habitats at three locations: Pigeon Point, Bean Hollow Beach, and Pescadero Point. They were taken to the laboratory in wet algae or aerated sea water and kept in aquaria containing filtered, aerated sea water at 13 ± 3° C. They were starved for one to three weeks prior to use to minimize the effect of varied nutritional state and contamination with fecal material. Oxygen consumption of several freshly collected animals was determined for comparison with oxygen consumption of the starved individuals.

Six sea urchins were sacrificed from each collection to determine the gonad index (wet weight of gonads divided by the wet weight of the body) as an indication of the reproductive condition of the population. For this purpose each animal was blotted on paper toweling for 30 seconds and weighed within ±0.1 gram on a triple beam balance. Then, following draining of the perivisceral fluid, the gonads were removed, blotted and weighed. In some cases the body wall indices were determined following removal of the Aristotle's lantern and the gut.

Oxygen electrode technique

The electrode used for monitoring the ambient oxygen partial pressure (AO_2) in the laboratory was a Beckman #315780 Clark electrode (Clark, 1956) coupled to the Beckman Model 160 Gas Analyzer (Beckman Instruments, Inc., Spinco Division, Palo Alto, California). This electrode was inserted through a sleeve of Tygon tubing in a 3/8-inch hole drilled in the Bakelite top of a specimen jar of desired size. This closed jar, immersed in a circulating water bath at the desired temperature, served as the respirometric chamber. As ambient oxygen was depleted by the sea urchin in the closed chamber, the Q_{O_2} (μl/g/hr) was calculated according to the equation: $Q_{O_2} = [\text{Vol. } O_2 \text{ consumed (ml/l)} \times \text{Vol. ambient water (l)}]/[\text{Times (hrs)} \times \text{Wet weight (g)}] \times 1{,}000$. The volume of O_2 consumed was calculated by converting the change in AO_2 in the vessel during the experiment from mm Hg to ml/l using the tables of Green and Carritt (1967).

A circulating water bath (Forma Scientific, Inc., #2095-2 refrigerated the external bath), was placed above the magnetic stirring unit. Water temperature was regulated to within 0.1° C in the respirometer chamber. The stirring bar placed below the test animal kept the ambient water in constant circulation during the experiment, equilibrating oxygen tension and temperature throughout the vessel (Figure 1).

Filtered sea water was used in the respirometric vessel, and was brought to equilibrium with air by gassing through a fine air stone for at least 30 minutes. Giese *et al.* (1966) showed microbial respiration to be a negligible factor at 13° C and below, so the use of antibiotics was suspended in all but the experiments at

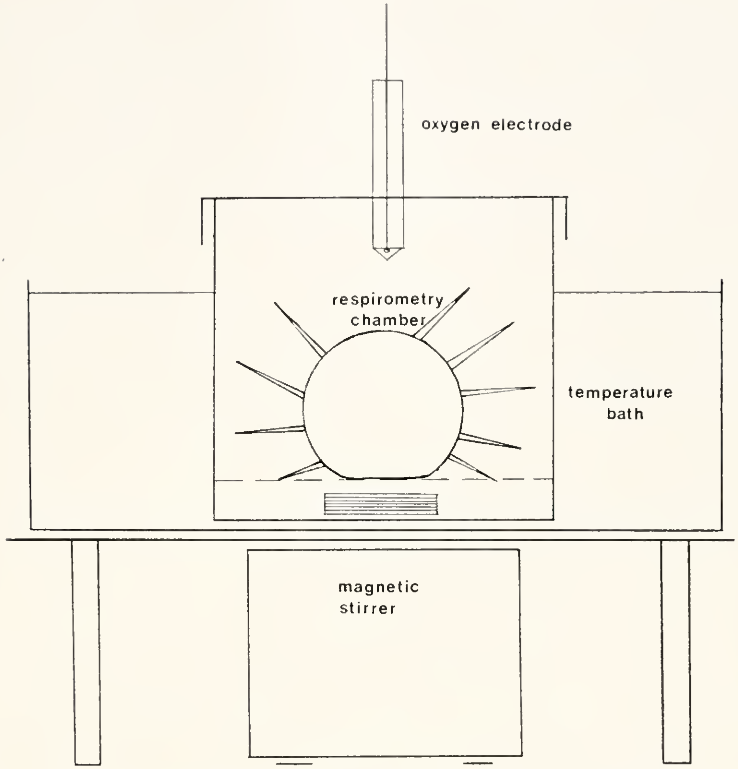


FIGURE 1. Representation of external water temperature bath and respirometric apparatus.

23° C. The oxygen consumption was about the same whether buffer was added to sea water or omitted (Lutz, 1930; Childress, 1968), though some carbon dioxide accumulation accompanied by a pH change occurs (Tang, 1933). Therefore buffering was also discontinued. The gas phase was eliminated from the chamber by the addition of sea water through the electrode port in the lid of the chamber prior to insertion of the electrode.

Chamber sizes were selected to provide a full-scale response (from 160 mm Hg to 0 mm Hg) in a period of five to six hours. Contrary to the findings of Johansen and Vadas (1967), our test animals failed to recover from exposure to an AO_2 below 80 mm Hg for more than one or two hours and generally died three or four days after the experiment. For this reason, only data from experiments of two hours duration are cited. Calibration of the electrode was checked before and after each run. If pre-experiment and post-experiment calibrations differed from each other by more than 5%, the data were discarded.

Prior to each oxygen depletion experiment, animals placed in the respirometric vessel were kept in aerated sea water for 15 to 30 minutes to achieve temperature equilibration with ambient sea water and to minimize the effects of possible excitation resulting from handling (Halcrow and Boyd, 1967; Childress, 1968).

Determinations of the ppO_2 of perivisceral fluid and gonad tissue were made with a Clark needle oxygen electrode (#17365), Instrumentation Laboratory, Lexington, Massachusetts. The electrode is constructed within a 4-3/4 inch long hollow stainless steel shaft of 0.2 mm diameter, and is covered with a tubular polypropylene membrane, closed at one end, containing a small amount of electrolyte gel. Because the electrode is of small diameter, is flexible, and is relatively rugged in its construction, it is ideally suited to investigations of this kind. The electrode was coupled to the Beckman Model 160 gas analyzer. Studies of tissue ppO_2 with this instrument must be analyzed with care, as electrophoretic protein deposition, while probing tissues, may "poison" the electrode and change its calibration (Silver, 1966). However, determinations of perivisceral fluid ppO_2, which has a low protein content, can be made without concern. The needle oxygen electrode was used in conjunction with an open chamber, which facilitated placement of the electrode into a known locus in the sea urchin. A 2 mm hole drilled in the test of the sea urchin allowed for the entry of the electrode into the perivisceral coelom. As clotting of the perivisceral fluid sealed the shaft of the electrode to the body wall, little exchange between perivisceral fluid and ambient sea water occurred through the hole.

Ambient oxygen partial pressures were regulated by gassing the surrounding sea water with air, oxygen, or nitrogen, and were monitored with a YSI Model 54 Oxygen Meter and YSI #5034 oxygen probe (Yellow Springs Instrument Company, Yellow Springs, Ohio).

EXPERIMENTAL RESULTS

Before determining the oxygen consumption during the reproductive cycle it was necessary to investigate the effects of some relevant variables on the rate of oxygen consumption. The present results were related to previous manometric studies by performing experiments in sequence on the same individuals with both manometry and oxygen electrodes.

Oxygen consumption at different ambient oxygen partial pressures

With an oxygen electrode in a closed system the ambient oxygen partial pressure (AO_2) falls as oxygen is consumed by the animal. It was therefore necessary to determine the effect of oxygen partial pressures (ppO_2) on oxygen consumption rate. A typical oxygen depletion curve for *S. purpuratus* (Figure 2 and Table I) indicates complete conformity between Q_{O_2} and ppO_2 to 5–10 mm Hg.

During the first two hours of each trial the AO_2 was depleted to the level of 80–100 mm Hg (Figure 2), equivalent to 3.0 to 3.7 ml/l at 13° C. Low environmental partial pressures of oxygen are rarely encountered by *S. purpuratus* in nature In fact the environmental partial pressures are seldom much below the air saturation level (Horne, 1969). An argument could thus be made for calculating the oxygen consumption rate on the basis of the earlier part of the depletion curve rather than the two hours chosen for this purpose, because the higher AO_2 more nearly represents the conditions to which the sea urchins are generally exposed in nature.

The oxygen consumption rates represented in Figure 3 (based on two-hour

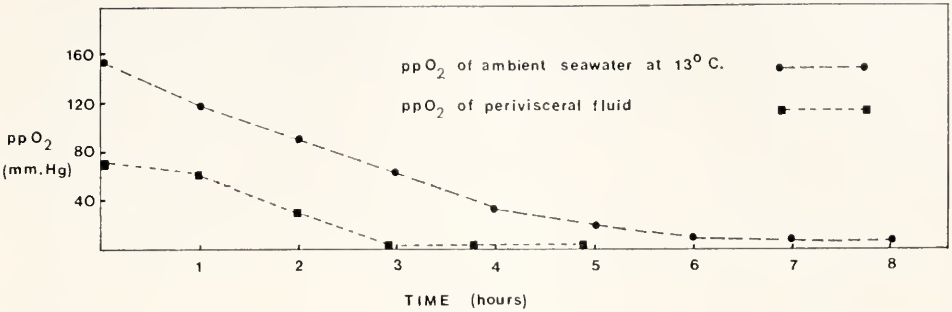


Figure 2. Typical oxygen depletion curve for *S. purpuratus* in a closed respirometer. Top line (dots) indicates ambient oxygen tension (AO_2) as a function of time. Bottom line (squares) represents perivisceral fluid oxygen tension ($PvfO_2$) during the same oxygen depletion run.

oxygen depletion experiments) are in essential agreement with the results of Johansen and Vadas (1967) and the oxygen depletion curves are similar to theirs of *S. purpuratus*. However, these authors did not indicate from which part of the oxygen depletion curve their Q_{O_2} values were calculated.

Comparison of manometry and oxygen electrodes

Oxygen consumption rates measured with Warburg manometry are uniformly higher than those obtained with the oxygen electrode system for equal sized sea urchins (Figure 3), probably because a constant AO_2 is maintained in the water by equilibration with the reservoir of air in the vessel, though possibly excitation occurs from the rocking of the manometers. Farmanfarmaian (1959) reports a constant rate of oxygen consumption throughout each two to three hour trial.

Seasonal and temperature background as it affects oxygen consumption

Data of seasonal effects on oxygen consumption in *S. purpuratus* have been collected only incidentally to other studies. Seasonal aspects of oxygen consumption might include the effects of factors considered later which change with season, such as temperature, nutrition, and reproductive state. Farmanfarmaian and

Table I

Q_{O_2} for three S. purpuratus *calculated for each hour during a five or six-hour run, on the basis of the mean ppO_2 in the chamber during each hour. All experiments at 13° C, Q_{O_2} in µl/g/hr.*

Hour	Q_{O_2} (31.5 g)	Q_{O_2} (67.1 g)	Q_{O_2} (89.0 g)
1	17.65	13.58	9.70
2	7.77	6.74	5.15
3	7.05	5.40	4.24
4	6.85	4.35	3.55
5	3.94	2.88	1.90
6		0.75	0.77

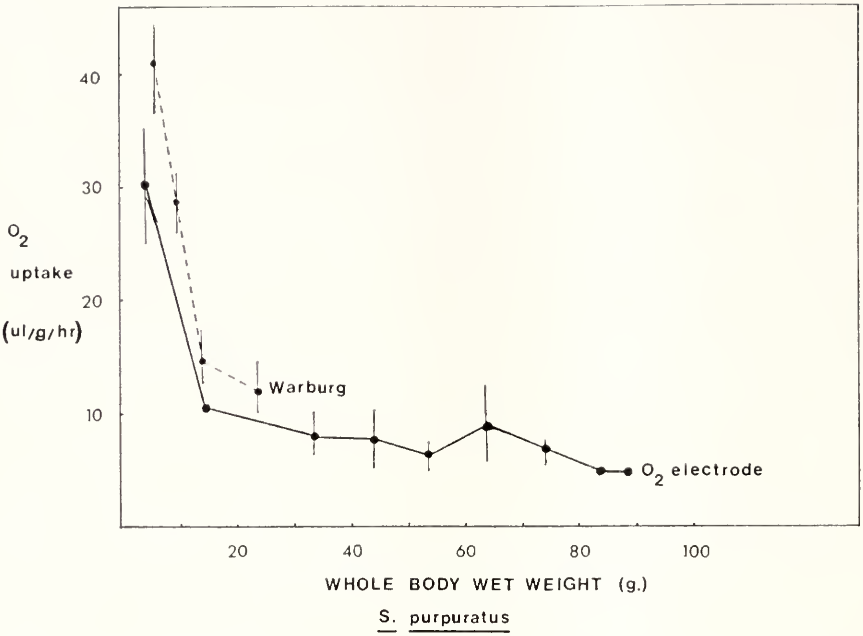


FIGURE 3. Oxygen consumption in *S. purpuratus* at 13° C as a function of whole body wet weight. Top line represents Warburg manometric data. Bottom line shows data obtained with oxygen electrode apparatus described in text. Vertical lines are single standard deviation units above and below the mean.

Giese (1963) found acclimation to lower temperatures (5° C) in the purple sea urchin over a period of 15 to 30 days, but no acclimation to higher temperatures, and the animals die when kept at temperatures above 23.5° C. Ulbricht and Pritchard (1972) found metabolic rate independence in *S. purpuratus* between 12 and 20° C. The present investigations were conducted at 13° C with sea urchins which had been held at that temperature for one to three weeks prior to use.

Tidal and circadian rhythms and oxygen consumption

Ulbricht and Pritchard (1972) studied the effect of tidal cycles and time of day on oxygen consumption in *S. purpuratus*, using oxygen depletion experiments of 36 hours' duration. They found no indication that tidal cycle or time of day affects the oxygen consumption rate. No evidence to the contrary was found in the present study in several experiments of 24 hours' duration.

Effect of oxygen enrichment on oxygen consumption

Two oxygen electrode studies demonstrated that oxygen consumption of *S. purpuratus* at ppO_2 above air saturation is greater than in air-saturated sea water. Thus when pure oxygen was bubbled through filtered sea water at 13° C to achieve a starting AO_2 of 390 to 400 mm Hg, 2-hour determinations with a 40.9 g

S. purpuratus yielded Q_{O_2} values of 18.4 to 18.6 μl/g/hr. AO_2 during the 2-hour run was depleted from 390 mm Hg to 290 mm Hg. These rates are about twice the Q_{O_2} for a 40 g *S. purpuratus* in air-saturated sea water at this temperautre (Figure 3).

Oxygen partial pressure of perivisceral fluid as a function of oxygen partial pressure of ambient sea water

Representative data obtained with a needle oxygen electrode (Figure 2) indicate a $PvfO_2$ between 40 and 60 mm Hg for 60 to 89 g *S. purpuratus* in sea water near air saturation (150 mm Hg). In a series of tests for sea urchins of similar size the $PvfO_2$ 13° C was found to vary between 23 and 54 mm Hg. In a series of tests on sea urchins of differing size, the $PvfO_2$ was found to increase directly with increase in size (Figure 4).

As the AO_2 falls during depletion of the oxygen in a closed vessel containing a sea urchin, the $PvfO_2$ changes little during the first 25 minutes following which it falls in parallel with the AO_2. $PvfO_2$ falls to zero when AO_2 falls to about 75–80 mm, *i.e.* about one half of the air-saturation level (Figure 2). With the needle oxygen electrode the initial temporary rise in $PvfO_2$ reported by Johansen and Vadas (1967) was not observed here.

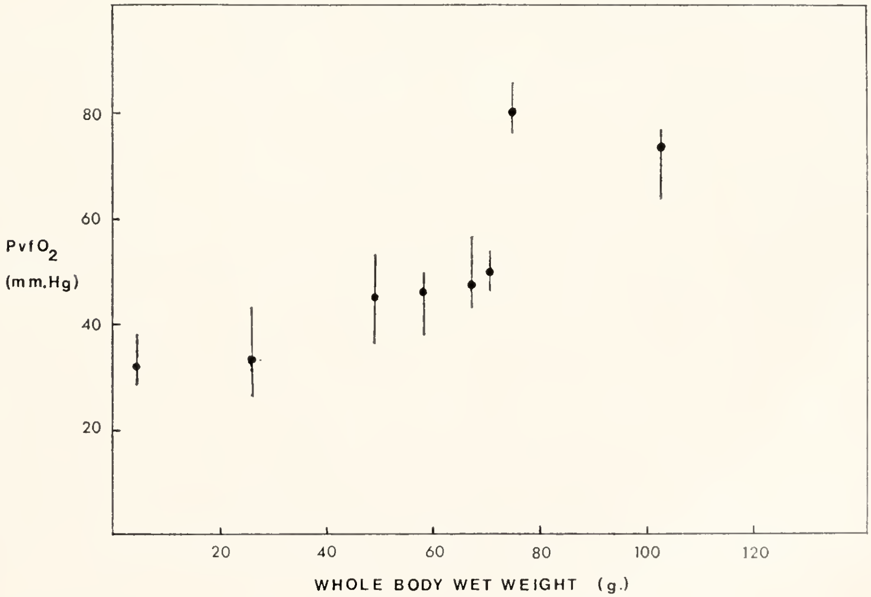


FIGURE 4. Perivisceral fluid ppO_2 in *S. purpuratus* as a function of whole body wet weight at 13° C. The coefficient of correlation (r) of 0.823 indicates a fairly high degree of relationship between these two factors. Points are mean PvO_2. High and low extremes during each experiment are indicated by vertical lines. Least squares analysis yields a y-intercept of 23.9, and a slope of 0.489.

Effect of body size on oxygen consumption

The relationship of oxygen consumption to body size in animals is usually expressed by the equation: metabolism = k·body weight^b where k and b are constants (Prosser and Brown, 1965; Newell, 1970). The value of b for larger metazoan poikilotherms is often quoted as 0.73, or intermediate between proportionality to weight and proportionality to surface area (Whitford and Hutchinson, 1967). This relationship is characteristic of most invertebrates (Zeuthen, 1947), including echinoderms (Farmanfarmaian, 1959, 1966).

Although total oxygen consumption is higher in larger *S. purpuratus,* the rate of oxygen consumption (expressed as Q_{O_2}) is higher for smaller animals than for their larger counterparts (Giese, 1966, Figure 3). The value of b for *S. purpuratus* over the wet weight range of 10 to 110 grams is approximately 0.65, a value in agreement with the findings of McPherson (1968) for *Eucidaris tribuloides.* As mentioned by Giese (1966), this makes body size (along with the nutritional state) an important factor to control in comparisons of oxygen consumption data for effects of different factors.

Oxygen consumption of isolated body wall

Using isolated body walls from *S. purpuratus* in the 10 to 100 g range, body wall oxygen consumption was found to be about four times the Q_{O_2} for the intact animal (Figure 5). Although the data are insufficient for statistical analysis, the Q_{O_2} for body wall tissue from small individuals was higher than that from large individuals (Table II). A decrease of the respiratory rates of the individual tissues, with an increase in size of the animal, may account for the lower Q_{O_2} of large as compared with small *S. purpuratus.* The Q_{O_2} of the dissected body wall may exceed the Q_{O_2} for the whole animal because both internal and external body wall surfaces are exposed to sea water near saturation with air, while only the external surface in the intact animal is so exposed; the internal surface of the intact animal is exposed to fluid containing only about one half to one fifth this much oxygen. Furthermore, the ambient fluid is circulated past the surface of the dissected body wall much more rapidly than in an intact animal, thereby considerably increasing the oxygen available to the tissue.

It is also possible that the isolated body wall had a higher oxygen consumption than in the intact animal because of a soluble "injury factor" diffusing from the dissected tissues. To test this the filtered and aerated supernatant from the crushed body wall leached in sea water for an hour was added to sea urchins. No detectable change in slope of the oxygen depletion curve was observed after such additions (three experiments). Thus, the Q_{O_2} for intact sea urchins before addition of extract averaged 10.2 μl/g/hr, while after addition of extract it averaged 10.4 μl/g/hr.

Effect of exposure of sea urchins to air

Since air contains 40 times as much oxygen as sea water, it might be reasoned that a sea urchin could survive in air provided it was kept moist. This appears

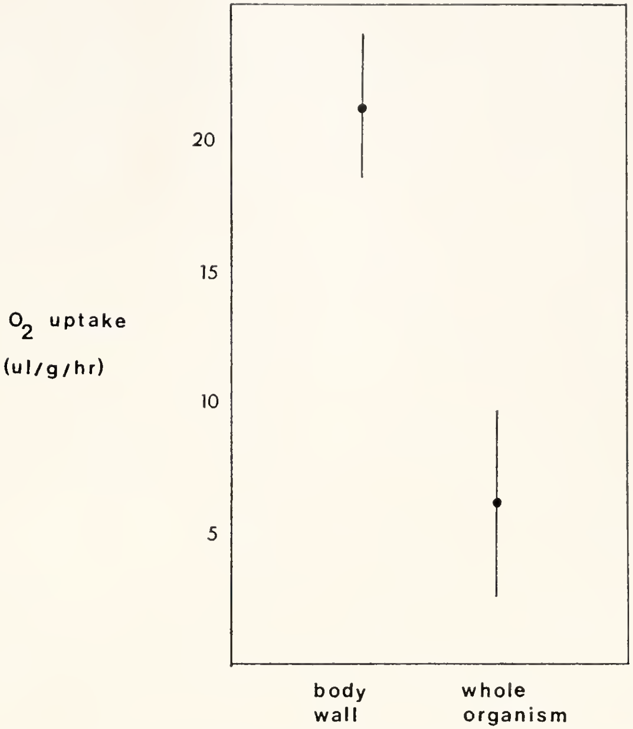


FIGURE 5. Comparison of oxygen consumption in intact *S. purpuratus* and the body wall component only; four trials at 13° C.

to be the case, although experiments with the oxygen needle electrode demonstrate that the $PvfO_2$ of sea urchins in air falls to almost zero after three hours exposure and remains there afterwards (Figure 6). This indicates the interruption of oxygen transport through the structures of the body wall of a sea urchin in air. These data are in agreement with those of Johansen and Vadas (1967) for *S. purpuratus.* Assuming no entry of oxygen from ambient sea water to ambulacral

TABLE II

Q_{O_2} (µl/g/hr) of the body wall as a function of body size (wet weight) in S. purpuratus *at 13° C.*

Wet weight (g)	Body wall index	Whole body Q_{O_2}	Body wall Q_{O_2}
10.5	61.5	28.6	39.0
62.9	44.2	11.1	30.7
72.3	41.2	6.49	24.9
83.0	44.2	5.78	23.4
94.6	48.3	4.57	18.2
96.1	46.3	8.85	18.2

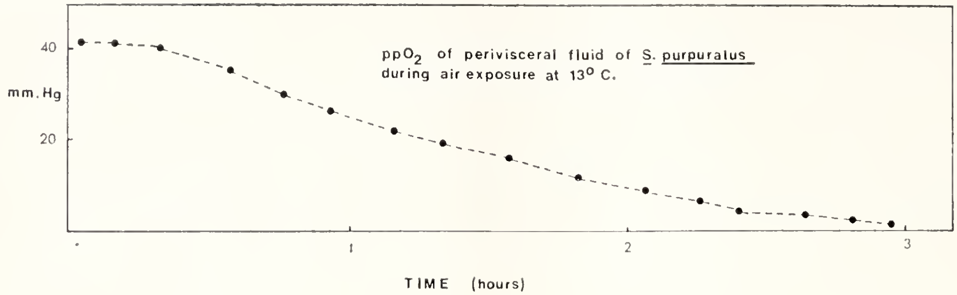


FIGURE 6. Needle electrode data indicating the change in perivisceral fluid oxygen tension with time in *S. purpuratus* in air at 13° C.

fluid during air exposure, on the basis of the oxygen depletion curve, the Q_{O_2} for the internal tissue is calculated to be 0.64 μl/g/hr. This is about one-tenth the expected Q_{O_2} for tissue of a sea urchin of this size, and is about one-half that for perivisceral fluid outside of the animal measured manometrically by Giese, Farmanfarmaian, Hilden and Doezema (1966).

Oxygen transport to the ambulacral system was stopped in another way by covering a sea urchin with Vaseline (Table III), a procedure which totally obstructs the exchange of oxygen with the body wall epithelium and tube feet (Farmanfarmaian, 1966). During this experiment the $PvfO_2$ was depleted from 40 mm Hg to zero in about 1.5 hours, yielding a Q_{O_2} for internal tissues of 1.18 μl/g/hr. This value and the value from sea urchins exposed to air indicate that the Q_{O_2} of the internal tissues probably accounts for no more than 10% of the total oxygen consumption of the intact *S. purpuratus.*

Although oxygen transport into the perivisceral coelom is interrupted during air exposure (Figure 6), the outer body wall obtains ample oxygen so long as the respiratory surface remains moist. Sea urchins used in the 3 hour air exposure experiments recover completely when returned to aquaria.

Oxygen consumption during the reproductive cycle

As measured by gonad index (Lasker and Giese, 1954) the reproductive cycle of *S. purpuratus* on the central California coast reaches its peak between November and January; spawnout occurs between January and March (Giese,

TABLE III

Comparison of the Q_{O_2} of internal tissues in the intact S. purpuratus *at 13° C, one animal in air, the other covered with Vaseline; Q_{O_2} for a whole animal in sea water at bottom.*

	Wet weight (g)	Q_{O_2}
Air exposure	46.8	0.64
Vaseline-covered	36.2	1.18
Whole body Q_{O_2} in sea water	38.7	8.9

1959). The gonad index reaches its lowest value between March and May. A maximal gonad index of about 25 has been measured for some members of a population near the peak, but the average for the present season near the peak was about 15; spawning is asynchronous—usually some individuals had spawned out when others were reaching the peak gonad size. The minimal gonad index varies between 3 and 5, but previous studies recorded values as low as 1.

The oxygen consumption of sea urchins was measured at various times during the reproductive cycle and the urchins were then dissected and the gonad index determined. The oxygen consumption rates were found to vary considerably for animals with both high and low gonad index (Table IV). Part of this variation is a result of the variation in size. However, animals of the same size and same gonad index varied considerably in their oxygen consumption. Since the factors considered to influence oxygen consumption were tested and taken into account in preparing for the present measurements, some factor other than those considered, and other than gonad index, must introduce the variability. That factor has remained elusive.

The major point of these experiments, however, was to determine whether the gonads were contributing appreciably to the oxygen consumption since they account for most of the doubling in organic matter in a gravid sea urchin. Tests of the gonads with the needle oxygen electrode at various times during the reproductive cycle in all cases gave ppO_2 values between 0.0 to 10.0 mm Hg (Figure 7). The method is inadequate to tell whether the higher values are for smaller gonads, because the same gonad gives different values on repeated trials.

Similarly, when measurements were made of the $PvfO_2$, an inverse correlation was found between $PvfO_2$ and weight of the sea urchin (Figure 4), rather than with gonad index, but the difficulties in making measurements with the oxygen micro electrode when the coelom is filled with tissue made it difficult to get reliable data for gravid sea urchins. The data are therefore mainly from specimens of low gonad index. These data indicate that the $PvfO_2$ is between 20 and 50% of the sea water saturation values of oxygen.

TABLE IV

The whole body Q_{O_2} as a function of gonad index in S. purpuratus *at 13° C. Least squares analysis gives Y intercept of 9.76 and a slope of −0.117, indicating little correlation between gonad index and oxygen consumption (Q_{O_2}).*

Gonad index	Wet weight	Q_{O_2}	Gonad index	Wet weight	Q_{O_2}
20.1	54.7	6.9	8.9	28.1	6.8
18.1	112.1	7.8	8.7	62.9	11.1
15.4	33.2	8.7	7.4	33.4	11.5
14.5	92.4	7.9	5.2	72.1	6.0
14.1	110.3	8.4	5.2	59.3	7.8
13.2	93.5	4.5	4.2	89.0	4.6
12.5	43.5	12.5	3.8	38.6	9.6
12.3	38.7	8.9	3.1	39.3	16.9
9.7	19.3	6.7	2.1	59.6	5.5
9.0	44.2	9.1	1.5	46.5	11.9

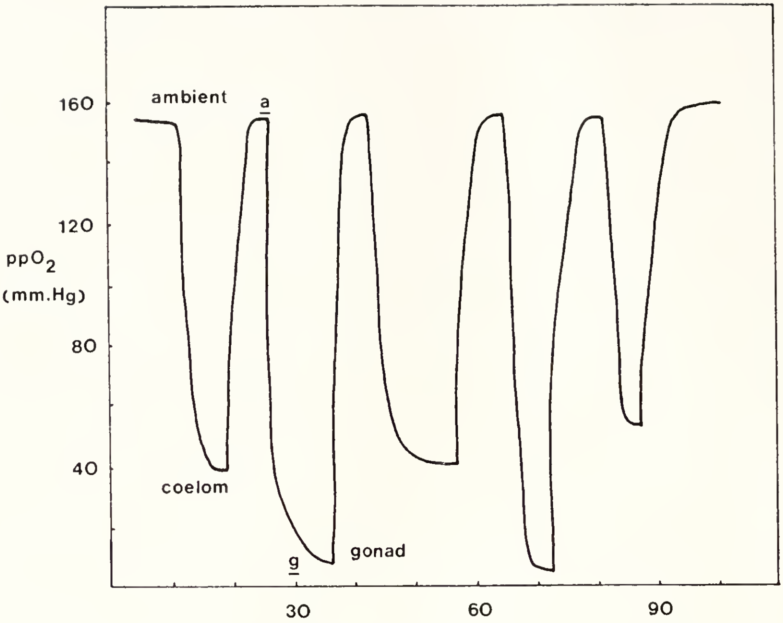


FIGURE 7. Representative data obtained with the needle oxygen electrode. Beginning with calibration in air saturation (157 mm Hg) the electrode is inserted through a hole drilled through the test into the perivisceral coelom. The perivisceral fluid oxygen partial pressure (PvO_2) is determined when a stable reading is achieved. The electrode is then withdrawn into ambient sea water and the calibration is checked (a). When stable at air saturation, the electrode is reinserted into gonad tissue (g), and a reading is made when a stable output is once again obtained. Two additional readings of PvO_2 and one of gonad tissue are represented.

DISCUSSION

The main purpose of the present study was to ascertain the reason for the finding that the rate of oxygen consumption (Q_{O_2}) of the purple sea urchin determined manometrically does not increase with increase in gonad size at the peak of the reproductive cycle when the total organic content of the body just about doubles (Giese, Farmanfarmaian, Hilden, and Doezema, 1966). The authors of the study quoted demonstrated that when the oxygen consumption of the body components was separately determined the summated Q_{O_2} for the entire body did increase with the increase in size of the gonads. They postulated that the limiting factor for such increased oxygen consumption in intact animals must be the lack of oxygen in the perivisceral fluid to supply the oxygen to the internal tissues. In other words, only the superficial cells in the large gonads were probably adequately supplied with oxygen and the bulk of the tissue had to take care of its metabolic needs by anaerobic processes.

The present study using oxygen electrode techniques corroborates the finding of a lack of correspondence between the size of the gonad and the rate of oxygen consumption which appears to depend mainly upon the size of the animal, other things being equal. The present study also supplies the information on the partial pressure of oxygen in the perivisceral fluid bathing the tissues, indicating that indeed it is low—at about 20 to 50% of the air-saturation value—and limits the rate of respiration of the internal tissues, which probably contribute only about 10 per cent of the total oxygen consumption of the sea urchin body. Unless oxygen is continuously supplied the partial pressure of oxygen falls as the tissues deplete it. Furthermore, measurements with a needle oxygen electrode demonstrate that the partial pressure of oxygen inside of tissues such as the gonad is indeed very low, between 10 mm Hg and zero. Such tissues are therefore internally anaerobic. Little is known of the anaerobic metabolism of tissues of the purple sea urchin, a study of which might be quite rewarding. Thus, regardless of the increase in mass and organic content (as measured by the total nitrogen of the gonads as they near the peak of the reproductive cycle), no corresponding oxygen consumption is found nor is it to be expected considering the limiting oxygen supply in the perivisceral fluid demonstrated here. It is possible that the variation in the measurements of perivisceral fluid oxygen may be a result of different locations of the electrode in the coelom. Owing to the poor circulation of the perivisceral fluid in the coelom, it is possible that next to the tissues where oxygen consumption occurs, the $PvfO_2$ is much lower than in the bulk of the fluid.

In the majority of the present experiments the oxygen depletion curve levels off before the last traces of oxygen have been consumed, a phenomenon also reported by Johansen and Vadas (1967) in *S. purpuratus* and *S. droebachiensis,* and by Mangum, Kushins and Sassaman (1970) in many invertebrate species. Apparently the animal has either reverted completely to anaerobic respiration at these low ambient oxygen partial pressures, or aerobic respiration is at such low levels as to be undetectable with the oxygen electrode.

The value for oxygen consumption of the purple sea urchin, *S. purpuratus,* determined by the oxygen electrode is somewhat lower than that determined by the manometric method, though both are of the same order of magnitude. The higher values for the manometric method are probably attributable to the higher oxygen partial pressure of the fluid continuously mixed with the air in the chamber. In the closed and fluid-filled chamber used with the oxygen electrode, the partial pressure of oxygen around the experimental animal declines at a rapid rate.

The present data provide information on a number of problems other than the relation between oxygen consumption and the reproductive cycle. Johansen and Vadas (1967) report that *S. purpuratus* regulates its oxygen consumption rate down to AO_2 levels of 60 to 70 mm Hg, and conclude that the Q_{O_2} is closely related to the ppO_2 of internal tissues and to the AO_2. They also consider the great variation of $PvfO_2$ of sea urchins in nature as evidence of metabolic regulation in *S. purpuratus,* but do not speak to the effect of body size on the $PvfO_2$. The results of this investigation contradict these statements. We find that $PvfO_2$ correlates inversely with the relative amount of body wall tissue (body wall index, Figure 4) in *S. purpuratus,* and therefore, with the Q_{O_2} of the intact animal. In other words, the $PvfO_2$ in small sea urchins is low in the presence of proportionally more body wall tissue of relatively high Q_{O_2} compared with large individuals. Apparently the important determinant of Q_{O_2} in *S. purpuratus* is the response of body wall tissue to the AO_2. Specimens of *S. purpuratus* and the dissected body wall component are both strict conformers and respire in direct proportion to the availability of environmental oxygen. The relatively high oxygen consumption of body wall tissue and its dependence upon environmental oxygen partial pressure determine the Q_{O_2} of the intact sea urchin; there is thus no need to invoke regulation.

Data concerning the recovery of *S. purpuratus* after periods of oxygen stress and air exposure may also be explained by high body wall respiration. During exposure to air the tube feet, normally extended during submergence, are collapsed. This greatly reduces the transport of oxygen across the tube feet to the water vascular system, and to the internal tissues. This subjects such tissues, which are adapted to low ppO_2, to almost anaerobic conditions. The outer surface of the body wall, while moist, and the inner surface—to the extent it is reached by diffusion of oxygen from the air—have abundant oxygen available during air exposure.

In contrast to total recovery from air exposure after return to air-saturated sea water, recovery of *S. purpuratus* from exposure to low ambient oxygen in sea water (below 80 mm Hg) for more than one or two hours is generally poor. Fewer than 10% of the *S. purpuratus* exposed to periods of oxygen stress remained alive for more than three days following their replacement into aerated aquaria. Periods of oxygen stress initiate the breakdown of the outer body wall in *S. purpuratus*, as evidence by the loss of spines, the lack of normal extension of the tube feet, and the release of echinochrome into the water.

SUMMARY

1. The rate of respiration (Q_{O_2}) of the sea urchin *Strongylocentrotus purpuratus* measured with an oxygen electrode parallels but is somewhat lower than that determined manometrically on the same individuals under the same conditions. The higher values of the Q_{O_2} obtained manometrically are attributable to the higher partial pressure of oxygen (ppO_2) in the fluid of the manometric vessels continuously equilibrated with air as compared to the closed chamber used with the oxygen electrode, in which the ppO_2 is continuously falling.

2. The effects of a number of factors on Q_{O_2} were determined: ppO_2 (including oxygen enrichment), relation between ambient oxygen partial pressure (AO_2), perivisceral oxygen partial pressure ($PvfO_2$) and effect of body size on Q_{O_2}, oxygen consumption of isolated and intact body wall; and the main thrust of this investigation—the change in oxygen consumption during the reproductive cycle.

3. The sea urchin is an oxygen conformer, its oxygen consumption being dependent upon the oxygen partial pressure from above-air saturated partial pressure following enrichment with oxygen down to 10 mm Hg. The oxygen partial pressure of the perivisceral fluid is also dependent upon the ambient oxygen partial pressure. The strict conformity of body wall tissue is the determining factor in the response of the whole body Q_{O_2} to ambient oxygen partial pressure. Internal oxygen partial pressures are the result of low internal oxygen availability after the body wall oxygen demand has been met, and do not determine the whole body Q_{O_2} in *S. purpuratus*.

4. *S. purpuratus* exhibits no systematic change in the Q_{O_2} as the reproductive cycle (as measured by the gonad index) reaches its peak, although the data

are quite variable. Relatively low perivisceral fluid oxygen partial pressures obtain throughout the year, and the gonadal tissue is under a nearly anaerobic condition at all times. The low oxygen availability to the gonadal tissues results in their having little input into the whole body Q_{O_2} of the sea urchin.

5. *S. purpuratus* cannot withstand ambient oxygen partial pressures below the 80 to 100 mm Hg level for more than one or two hours due, apparently, to the sensitivity of the body wall to attenuated ambient oxygen. This is an important factor in restricting this species to habitats near the air saturation level of ppO_2.

LITERATURE CITED

CHILDRESS, J., 1968. The respiratory physiology of the oxygen minimum layer mysid, *Gnathophausia ingens. Doctoral dissertation, Stanford University,* 142 pp.

CLARK, L. C., 1956. Monitor and control of blood and tissue oxygen tension. *Trans. Soc. for Art. Int. Organs,* **2**: 41.

FARMANFARMAIAN, A. A., 1959. The respiratory surface of the purple sea urchin, *Strongylocentrotus purpuratus. Doctoral dissertation, Stanford University,* 98 pp.

FARMANFARMAIAN, A., 1966. The respiratory physiology of echinoderms. Pages 245–265 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience Publishers, New York.

FARMANFARMAIAN, A. A., AND A. C. GIESE, 1963. Thermal tolerance and acclimation in the western purple sea urchin, *Strongylocentrotus purpuratus. Physiol. Zool.,* **36**: 237–243.

GIESE, A. C., 1959. Annual reproductive cycles of marine invertebrates. *Annu. Rev. Physiol.,* **21**: 547–576.

GIESE, A. C., 1966. Changes in body component indices and respiration with size in the purple sea urchin, *Strongylocentrotus purpuratus. Physiol. Zool.,* **40**: 194–200.

GIESE, A. C., A. FARMANFARMAIAN, S. HILDEN AND P. DOEZEMA, 1966. Respiration during the reproductive cycle in the sea urchin, *Strongylocentrotus purpuratus. Biol. Bull.,* **130**: 192–201.

GREEN, E. J., AND D. E. CARRITT, 1967. New tables for the oxygen saturation of sea water. *J. Mar. Res.,* **25**: 140–147.

HALCROW, K., AND C. M. BOYD, 1967. The oxygen consumption and swimming activity of the amphipod *Gammarus oceanicus* at different temperatures. *Comp. Biochem. Physiol.,* **23**: 233–242.

HORNE, R. A., 1969. *Marine Chemistry.* Wiley-Interscience, John Wiley and Sons, New York, 568 pp.

JOHANSEN, K., AND R. L. VADAS, 1967. Oxygen uptake and responses to respiratory stress in sea urchins. *Biol. Bull.,* **132**: 16–22.

LASKER, R., AND A. C. GIESE, 1954. Nutrition of the sea urchin, *Strongylocentrotus purpuratus. Biol. Bull.,* **106**: 328–340.

LUTZ, B. R., 1930. The effect of low oxygen tension on pulsations of the isolated holothurian cloaca. *Biol. Bull.,* **58**: 74–84.

MCPHERSON, B. F., 1968. Feeding and oxygen uptake of the tropical sea urchin *Eucidaris tribuloides* (L.). *Biol. Bull.,* **136**: 308–320.

MANGUM, C. P., L. J. KUSHINS AND C. SASSAMAN, 1970. Responses of intertidal invertebrates to low oxygen conditions. *Amer. Zool.,* **10**: 516–517.

NEWELL, R. C., 1970. *The Biology of Intertidal Animals.* American Elsevier Pub. Co., New York, 555 pp.

PROSSER, C. L., AND F. A. BROWN, JR., 1965. *Comparative Animal Physiology.* W. B. Saunders Co., Philadelphia, 688 pp.

SILVER, I. A., 1966. Pages 135–153 in *Oxygen Measurements in Blood and Tissues,* J. P. Payne and D. W. Hill, Eds., Little, Brown and Co., Boston.

STEEN, J. B., 1965. Comparative aspects of respiratory gas exchange in sea urchins. *Acta Physiol. Scand.,* **63**: 164–170.

TANG, P. S., 1933. Oxygen consumption as a function of oxygen pressure. *Quart. Rev. Biol.,* **8**: 260–274.

ULBRICHT, R. J., AND A. W. PRITCHARD, 1972. Effect of temperature on the metabolic rate of sea urchins. *Biol. Bull.,* **142**: 178–185.

WHITFORD, W. G., AND V. H. HUTCHINSON, 1967. Body size and metabolic rate in salamanders. *Physiol. Zool.,* **40**: 127–133.

ZEUTHEN, E., 1947. Body size and metabolic rate in the animal kingdom. Oxygen uptake as related to body size in organisms. *Quart. Rev. Biol.,* **28**: 1–12.

Continued from Cover Two

4. ***Literature Cited.*** The list of references should be headed LITERATURE CITED, should conform in punctuation and arrangement to the style of recent issues of THE BIOLOGICAL BULLETIN, and must be typed *double-spaced* on separate pages. Note that citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS of those abstracted (most recent issue: November, 1972). Foreign authors, and others who are accustomed to use THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. at £0.65 or $1.75) useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (not strictly as USASI usage, *i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.* not *J.Cell.Comp.Physiol.*)

E. We strongly recommend that more unusual words in journal titles be spelled out in full, rather than employing lengthy, peculiar "abbreviations" or new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation. Even in more familiar languages, *Z. Vererbungslehre* is preferred to *Z. VererbLehre* (WORLD LIST) or *Z. Vererbungsl.* (USASI). *Accurate and complete communication of the reference is more important than minor savings in printing costs.*

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

H. Spell out *London, Tokyo, Paris, Edinburgh, Lisbon, etc.* where part of journal title.

I. Series letters *etc.* immediately before volume number.

J. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond.; Science, N.Y.; Evolution, Lancaster, Pa.*)

K. The correct abbreviation for THE BIOLOGICAL BULLETIN is *Biol. Bull.*

5. ***Figures.*** The dimensions of the printed page, 5 by 7⅜ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction. Explanatory matter should be included separately in legends as far as possible, although the axes should always be numbered and identified on the illustration itself. Figures should be prepared for reproduction as either line-cuts or halftones; no other methods will be used. Figures to be reproduced as line-cuts should be drawn in black ink on white paper, good quality tracing cloth or plastic, or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on board, and both designating numbers or letters and scale-bars should be affixed directly on the figures. We recommend that halftones submitted to us be mounted prints made at about 1½ times the linear dimensions of the final printing desired (the actual best reductions are achieved from copy in the range from 1¼ to 2 times the linear dimensions). As regards line-blocks, originals can be designed for even greater reductions but are best in the range 1½ to 3 times. *All* figures should be numbered in consecutive order, with no distinction between text and plate-figures. The author's name should appear on the reverse side of all figures, and the inked originals for line-blocks must be submitted for block-making.

6. ***Mailing.*** Manuscripts should be packed flat. All illustrations larger than 8½ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.

Reprints. Reprints may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

CONTENTS

Betzer, Susan B. and Michael E. Q. Pilson
Copper uptake and excretion by *Busycon canaliculatum* L. 1

Betzer, Susan B. and Paul P. Yevich
Copper toxicity in *Busycon canaliculatum* L. 16

Bradley, Brian P.
The anomalous influence of salinity on temperature tolerances of summer and winter populations of the copepod *Eurytemora affinis* . . 26

Bush, Louise
Biology of *Neochildia fusca* n. gen., n. sp. from the northeastern coast of the United States (Platyhelminthes: Turbellaria) 35

Komatsu, Miéko
On the development of the sea-star, *Astropecten latespinosus* Meissner 49

Kruczynski, W. L.
A radioactive tracer study of food uptake by *Pinnotheres maculatus* in molluscan hosts 60

Polls, Irwin and J. Gonor
Behavioral aspects of righting in two asteroids from the Pacific Coast of North America 68

Roberts, John L.
Active branchial and ram gill ventilation in fishes 85

Sehnal, Frantisek and Noelle A. Granger
Control of corpora allata function in larvae of *Galleria mellonella* . . 106

Shick, J. Malcolm
Uptake and utilization of dissolved glycine by *Aurelia aurita* scyphistomae: temperature effects on the uptake process; nutritional role of dissolved amino acids 117

Stefano, George B. and Edward Aiello
Histofluorescent localization of serotonin and dopamine in the nervous system and gill of *Mytilus edulis* (Bivalvia) 141

Webster, Steven K.
Oxygen consumption in echinoderms from serveral geographical locations, with particular reference to the Echinoidea 157

Webster, Steven K. and Arthur C. Giese
Oxygen consumption of the purple sea urchin with special reference to the reproductive cycle 165

Volume 148 Number 2

THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

JOHN M. ANDERSON, Cornell University
JOHN B. BUCK, National Institutes of Health
JOHN O. CORLISS, University of Maryland
JOHN D. COSTLOW, Duke University
CATHERINE HENLEY, University of North Carolina
GEORGE O. MACKIE, University of Victoria
F. H. RUDDLE, Yale University
BERTA SCHARRER, Albert Einstein College of Medicine
HOWARD A. SCHNEIDERMAN, University of California, Irvine
GROVER C. STEPHENS, University of California, Irvine
CARROLL M. WILLIAMS, Harvard University
EDWARD O. WILSON, Harvard University

W. D. RUSSELL-HUNTER, Syracuse University
Managing Editor

APRIL, 1975

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

b

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $7.00. Subscription per volume (three issues), $18.00, (this is $36.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between May 23 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

Copyright © 1975, by the Marine Biological Laboratory

Second-class-postage paid at Lancaster, Pa.

INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. *A condensed title* or running head of no more than 35 letters and spaces should be included.

Continued on Cover Three

ERRATUM

THE BIOLOGICAL BULLETIN, Volume 147, Number 3, Page 679.

The following corrected version of Figure 1 should be substituted for Figure 1 as printed on page 679, in the paper by Graham Walker entitled, "The occurrence, distribution and attachment of the pedunculate barnacle *Octolasmis mülleri* (Coker) on the gills of crabs, particularly the blue crab, *Callinectes sapidus* Rathbun" (1974, *Biol. Bull.*, 147: 678–689).

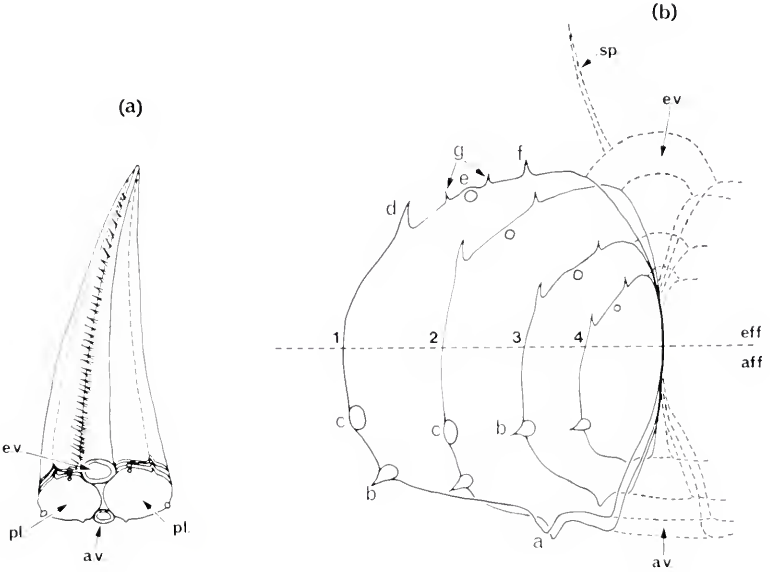


FIGURE 1. (a) A drawing of the 4th gill of *Callinectes sapidus* showing the paired platelets (pl.), small afferent vessel (a.v.) and larger efferent vessel (e.v.); (b) drawing of gill platelets taken from different regions (1, 2, 3, 4) of the same gill. The platelets are divided into afferent (aff.) and efferent (eff.) zones and show the marginal projections: spines a, b, d, f, g and knobs c and e; a.v., afferent vessel; e.v., efferent vessel; sp., spine.

Vol. 148, No. 2 April, 1975

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

SURFACE ULTRASTRUCTURE OF THE SCYPHOPOLYP, *CHRYSAORA QUINQUECIRRHA*

Reference: *Biol. Bull.,* **148**: 181-192. (April, 1975)

RICHARD S. BLANQUET AND BRUCE WETZEL

Department of Biology, Georgetown University, Washington, D. C. 20007, and Dermatology Branch, National Cancer Institute, Bethesda, Maryland 20014

Cnidarians are aquatic animals thought to represent the simplest and perhaps basic structural organization of multicellular, metazoan organisms. The body plan consists of a blind sac with a single opening, the mouth, surrounded by tentacles. The body wall consists of two cell layers; an outer epidermis and an inner gastrodermis separated by a gelatinous mesoglea of varying thickness and cellularity. The enclosed space is referred to as the gastrocoel or gastrovascular cavity. Water in the gastrocoel is occasionally flushed by periodic body contractions and, in many cases, continually by currents generated by ciliated gastrodermal cells. Cells of both epithelial layers thus have direct contact with the external environment.

In diploblastic organisms, functional specialization involves the differential distribution of various cell types, as well as structural specialization of the surface of individual cells. Despite the importance of cell interaction with the environment in cnidarian function, little consideration has been given to surface ultrastructure in contrast to the extensive literature dealing with various other aspects of the relatively few cnidarian cell types. Furthermore, most of these studies represent the classes Hydrozoa and Anthozoa. Therefore, this study was undertaken to characterize the surface features of a Scyphozoan polyp, *Chrysaora quinquecirrha* by scanning electron microscopy and, when possible, to relate such structure to function.

MATERIALS AND METHODS

The polyps used in this study were obtained from populations of *Chrysaora quinquecirrha* maintained in culture in the laboratory. Specimens were prepared for scanning electron microscopy (SEM) by fixation at room temperature in 5% glutaraldehyde made up with sea water (10‰). Polyps were rinsed in tap water for 30 minutes, dehydrated through a graded ethanol series to isoamylacetate, and dried by the critical point method with carbon dioxide. For observation of gastro-

Copyright © 1975, by the Marine Biological Laboratory
Library of Congress Card No. A38-518

dermal structures, some dried polyps were sectioned longitudinally with microforceps and a razor blade. The animals were then affixed to specimen stubs with scotch (3M) transfer tape, coated with carbon and gold-palladium, and examined with an Etec Autoscan electron microscope operated at 45° tilt and at 20 kV unless otherwise indicated.

Observations on living specimens were made using bright field and phase contrast microscopy. Studies of currents generated by ciliary motion were facilitated by the addition of a dilute suspension of carmine particles. Gastrodermal currents were observed in animals which were cut in half lengthwise with a razor blade.

Results

Chrysaora polyps are goblet-shaped organisms which attach to hard substrata by means of an aboral pedal disc (Fig. 1). A slender body region, the stalk, extends from this point of attachment and then widens abruptly to form the calyx. The oral disc (Fig. 2), with its centrally located mouth and surrounded by a single row of tentacles, forms the distal end.

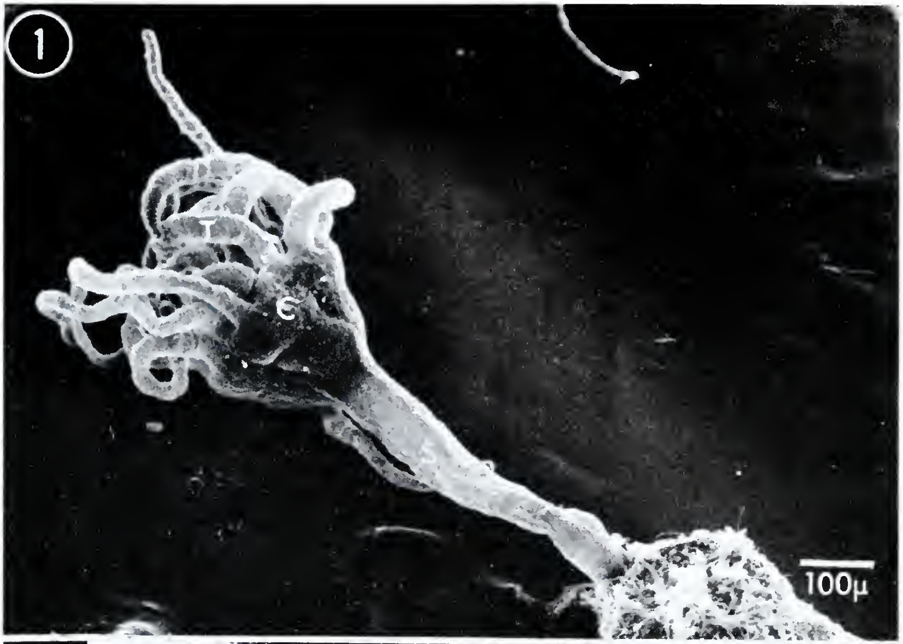
Epidermis

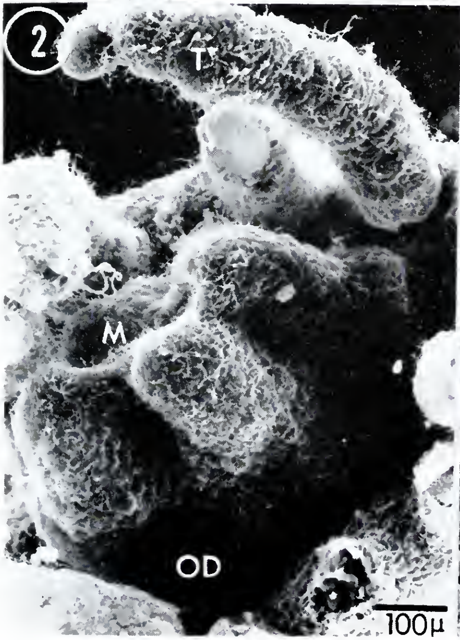
All regions of the epidermis are ciliated. A moderate number of cilia appear on the oral disc and epithelium between the tentacles (Figs. 2, 3). In contrast, the upper calyx is more heavily ciliated (Fig. 4) with the incidence of cilia decreasing toward the lower portion of the calyx (Fig. 5) and along the stalk toward the pedal disc.

Most cells possess a single cilium surrounded at the base by a circlet of small, closely spaced microvilli (Figs. 5, 6). The cilia on the oral disc arise from a crater formed by the fusion of 9–12 microvilli (Fig. 6) and are highly motile. Cilia of this nature are also common on the calyx and tentacles (Figs. 7, 8). In addition to those surrounding the cilia, epidermal cells of tentacles, calyx and stalk possess numerous, scattered microvilli (Figs. 5, 7). Fine, filamentous strands which occur between microvilli (Fig. 5) are thought to represent mucus preserved during fixation. There is a conspicuous paucity of microvilli on the oral disc (Figs. 3, 6).

Other cilia arise from a broad, conical base (Fig. 8) probably consisting of fused microvilli. These cilia are most abundant on the tentacles. They are present to a lesser extent on the calyx, and they are occasionally seen on the stalk. The distribution of these broad-based cilia corresponds to the distribution of nematocysts observed by light microscopy in living polyps. Discharged nematocyst threads originate in close proximity to the broad, conical bases (Figs. 9, 10) and thus these cilia are considered to be cnidocils. As viewed by phase microscopy in living animals, cnidocils appear as long, fairly straight structures which vibrate slightly in the current generated by the motile epithelial cilia.

Two types of nematocysts, microbasic euryteles and atrichous isorhizas (nomenclature according to Weill 1930, 1934), are abundant on the tentacles and to a lesser extent on the calyx. Atrichous isorhizas actually possess small, uniformly distributed barbs along the dischaged thread (Figs. 7, 9, 10). Tentacle nematocysts can be made to discharge when the polyp is placed in 0.5% acidified methylene





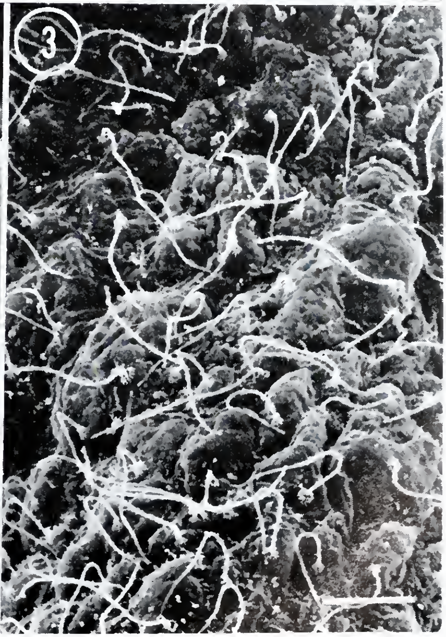
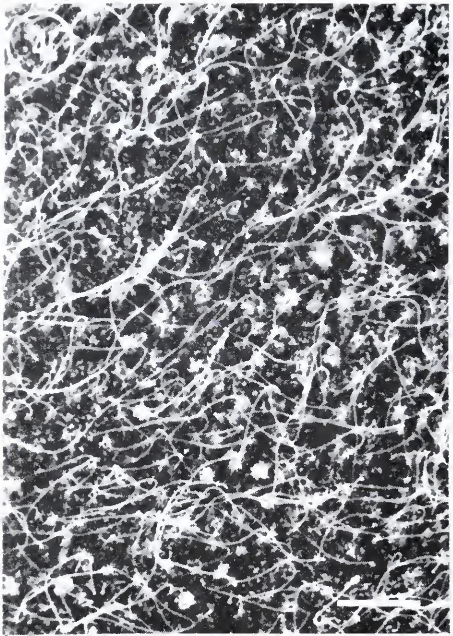
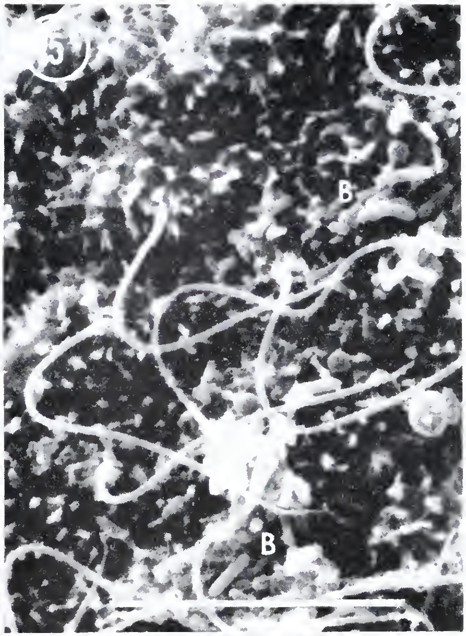


FIGURE 1. *Chrysaora* polyp illustrating the major body regions; tentacles (T), calyx (C) and stalk (S).

FIGURE 2. Oral disc (OD) revealed by removal of most surrounding tentacles (T). The mouth (M) is centered in a raised hypostome with four radial folds.

FIGURE 3. Oral disc showing sparse ciliation and few microvilli on the smooth cell surfaces. Compare with the heavily ciliated inner surface of the oral disc (Fig. 12); 10 µm scale marker.





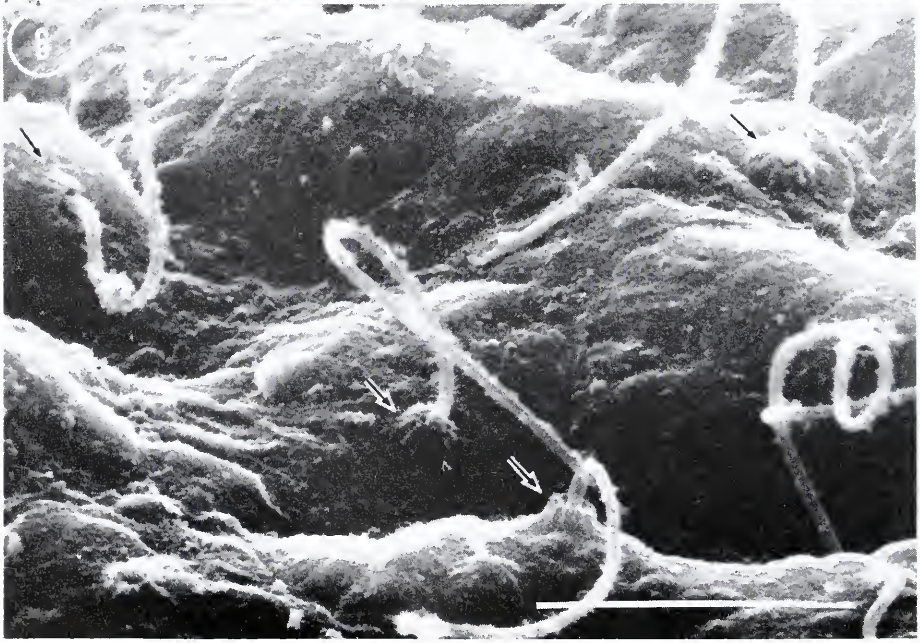


FIGURE 4. Heavily ciliated region of the upper calyx. Most cilia are curved in the same direction suggestive of ciliary movement and consistent with the rapid current observed in this area; 10 μm scale marker.

FIGURE 5. Surface of lower calyx with fewer cilia and numerous microvilli. Microvilli are loosely clustered around the base of each cilium. Note the numerous bacteria (B) adhering to

blue. Nematocysts in the calyx also discharge under these conditions and thus are probably functional on the body as well.

Gastrodermis

In scyphopolyps, the gastrocoel is divided by four septae which extend from the region of the oral disc to the junction of the calyx and stalk (Fig. 11). Of particular interest is the nature of the epithelium which lines the oral disc and the free edges of the septae. This epithelium has been referred to by Chapman (1966) as the scyphopharynx-filament complex (SPC) and its distinctive appearance in comparison to other regions of the polyp has long been recognized (Tcheou-Tai-Chuin, 1930).

The SPC is the most heavily ciliated region of the polyp (Figs. 12, 13). Most cilia arise from circlets of short, fused microvilli. Many cilia in this region, especially along the septal edge, are surrounded by rather long, fused microvilli (Fig. 14) which form broad conical bases like those which distinguish tentacle cnidocils. Nematocysts, mainly microbasic euryteles, are abundant on the septae, particularly along the free edge. Numerous microvilli are interspersed among the cilia.

Large, smooth, dome-shaped structures, approximately 3μm in size, and surrounded by microvilli (Fig. 14), occur on the septal epithelium and inner surface of the oral disc. These structures may represent nematocyst sites or the release of secretory material. Cells of the SPC are capable of ingesting small particles as evidenced by the ingestion of carmine particles which remain in such cells for several days afterwards.

Gastrodermal cells (Fig. 15) which constitute the remainder of the gastrocoel lining possess a single cilium and extremely abundant, long microvilli consistent with their absorptive function.

Ciliary currents

Bright field and phase contrast microscopy reveals that cilia associated with epidermal and gastrodermal cells are highly motile. Motile epidermal cilia generate a vigorous current which flows distally from the pedal disc, along the body column, tentacles and oral disc, carrying small particles away from the organism (Fig. 16). A similar current exists along the gastrodermal epithelium (Fig. 17). Carmine particles falling on the gastric septae and body wall are carried toward the mouth. The current is particularly rapid along the septal edge.

Attempts to reverse the ciliary beat either mechanically or chemically by feeding activators were unsuccessful. The addition of *Artemia* extracts or a 10^{-5} M reduced glutathione solution resulted in a typical feeding response (Loeb & Blanquet, 1973) with no reversal of either external or internal currents. During

---

the surface and the fine, filamentous strands which extend between microvilli; 10 μm scale marker.

FIGURE 6. Epithelial surface of the oral disc. Cilia arise from craters formed by a circlet of short, fused microvilli. Few microvilli occur elsewhere on the cell surface; 10 μm scale marker.

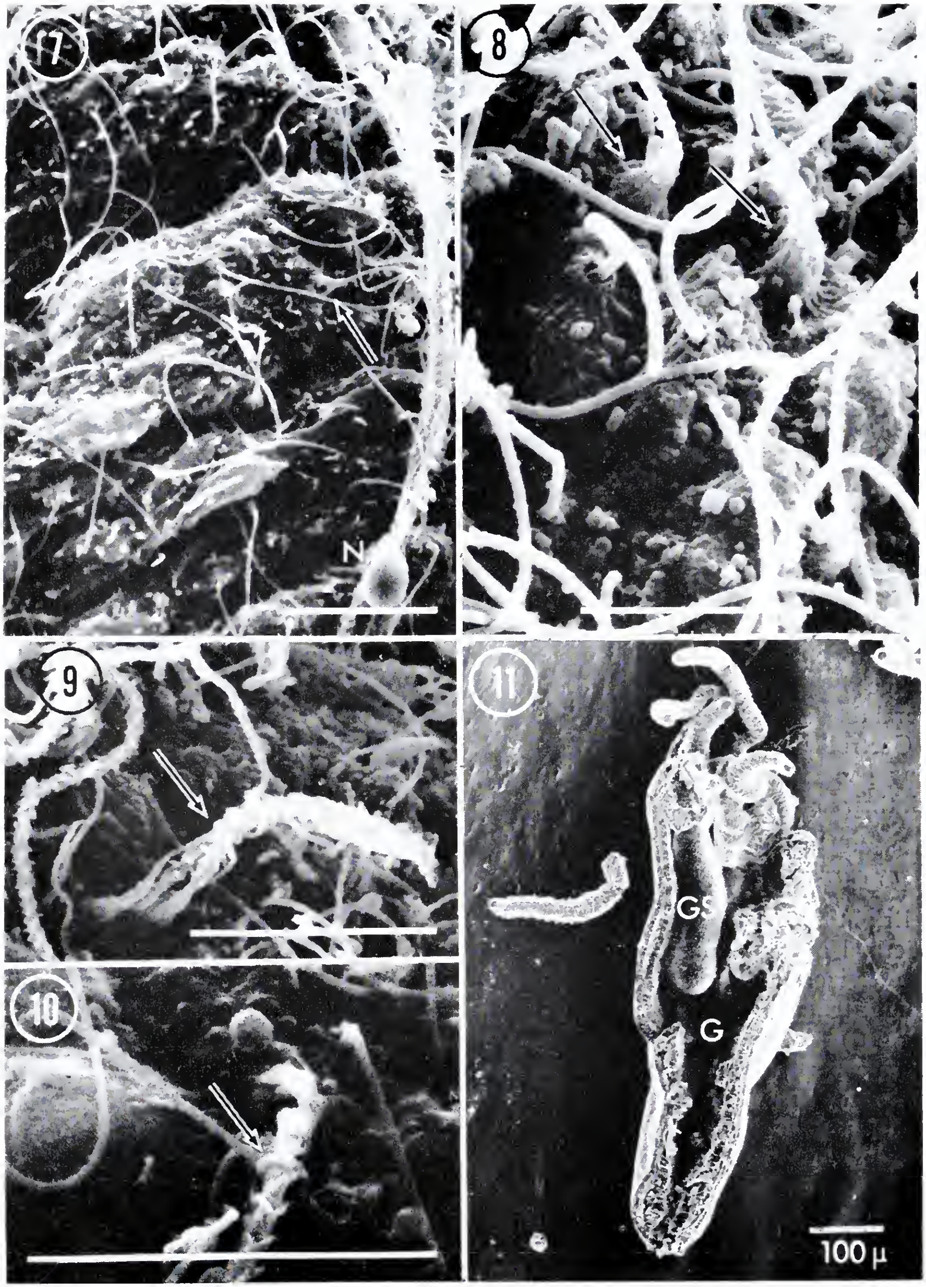


FIGURE 7. Surface of tentacle showing the distribution of cilia and microvilli. A free nematocyst (N, holotrichous isorhiza) with its everted, barbed thread is present. Discharged threads of unliberated nematocysts are also seen. Numerous broad-based cnidocils appear on the tentacle surface (*e.g.*, arrow); 10 μm scale marker.

feeding, the tentacles bend toward the mouth and are frequently inserted into the gastrocoel. In this position, small particles may be swept into the gastrocoel by the current along the tentacles. These are caught almost immediately in the outward flow and are carried away. Thus, in both feeding and non-feeding situations, small particles move away from the polyp.

Discussion

The scyphopolyp, or scyphistoma, represents the asexual reproductive stage in the dimorphic life cycle of many scyphozoan medusae. Such polyps are structurally more complex than hydrozoan polyps but less complex than anthozoan polyps. Compared to the extensive research carried out on hydrozoan and anthozoan polyps, little work has been done on scyphistomae.

Consideration of cell surface interaction with the environment is essential to an understanding of cnidarian function. Scanning electron microscopy (SEM) is ideally suited to survey the surfaces of small organisms. The excellent resolution, increased depth of field and the three dimensional effect of SEM images enables the study of the detailed morphology and distribution of fine surface features. To our knowledge, however, only one survey of both the epidermal and gastrodermal epithelia of a cnidarian polyp has been reported (Beams, Kessel and Shih, 1973). This work concerned the fresh water polyp, *Hydra,* and we believe the present investigation on *Chrysaora* is the first such study on a scyphozoan polyp. SEM enables a better integration of data from light and transmission electron microscopy and allows a more accurate correlation of structure and function. This is of value in comparative studies and in the understanding of the structural adaptation of polyps to different environments.

In marked contrast to *Hydra,* all regions of *Chrysaora* are extensively ciliated. The appearance and arrangement of microvilli around the bases of *Chrysaora* cilia varies; these microvillar configurations being distributed differentially over the polyp. In *Chrysaora,* the tentacles, upper calyx, and especially the scyphopharyngeal-filament complex (SPC) represent the most heavily ciliated regions of the polyp. As these regions are most likely to receive external stimuli, the distribution of cilia is consistent with the possible function of at least some as sensory receptors. A well developed sensory capability would especially benefit an estuarine organism such as *Chrysaora* which is subject to a constantly changing environment.

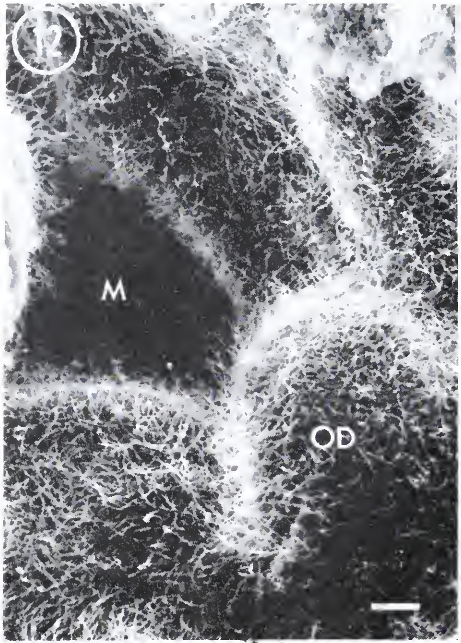
Sensory cilia have been reported in numerous cnidarians (McConnell, 1932; Mackie, 1960; Burnett and Diehl, 1964; Lentz and Barrnett, 1965; Jha and Mackie, 1967). Josephson (1961) has demonstrated that certain hydroids are

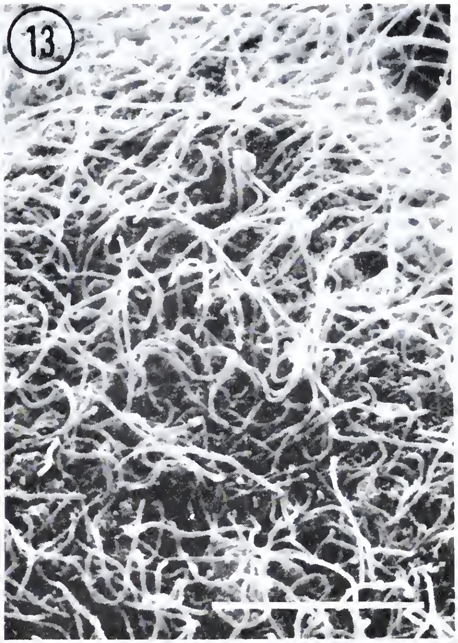
---

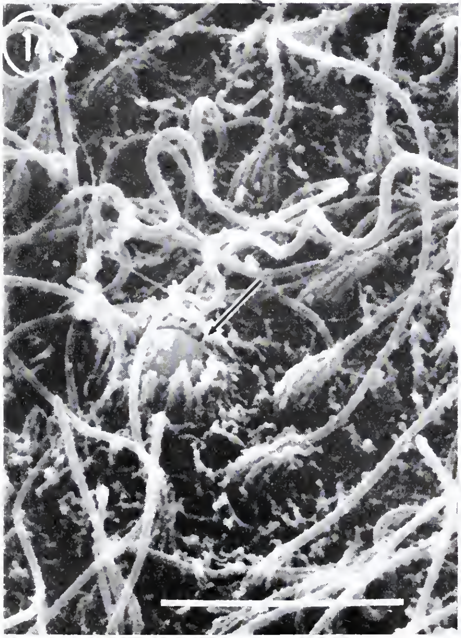
FIGURE 8. Detailed view of the broad conical bases of tentacle cnidocils (arrows). These clearly differ from the circlets of short microvilli surrounding the cilia immediately below them; 10 μm scale marker.

FIGURE 9, 10. Surface of tentacle depicting the close association of broad-based cnidocils with discharged nematocyst threads. Note that the cnidocil shafts are entwined around the discharged threads; 10 μm scale marker.

FIGURE 11. Polyp bisected longitudinally to reveal the gastrocoel (G) and one fold of the prominent septae (GS). The diploblastic nature of the polyp is readily apparent in the wall of the calyx.







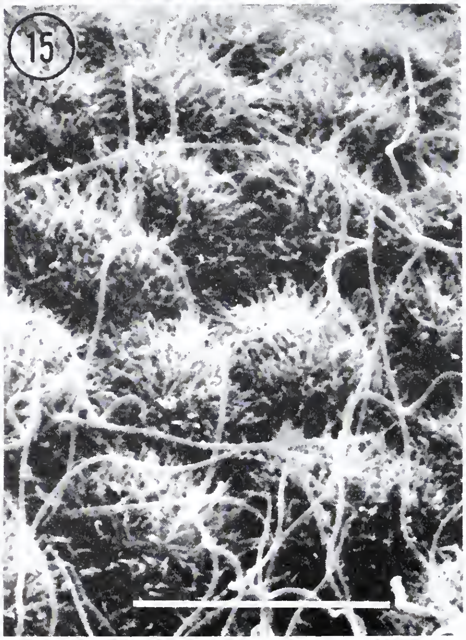


FIGURE 12. Mouth (M) and inner surface of the oral disc (OD) as seen from within the gastrocoel. This is the most heavily ciliated region of the polyp; 10 μm scale marker.

FIGURE 13. Detailed view of the inner surface of the oral disc. Cilia are so numerous they obscure the cell surface; 10 μm scale marker.

BIOLOGICAL LABOR

responsive to weak water-borne stimuli. Cilia have been shown to act as receptors for such stimuli in ctenophores and chaetognaths (Horridge, 1965; Horridge and Boulton, 1967). Cilia are also associated with cnidarian statocysts (Horridge, 1969) and photoreceptors (Horridge, 1969; Eakin and Westfall, 1962).

Transmission electron microscopy has shown that cnidocils are modified cilia which arise from a broad base of fused microvilli or sterocilia (Chapman and Tilney, 1959a, b; Lentz, 1966; Westfall, 1965, 1970). The broad-based cilia described in the present study as cnidocils correspond to these descriptions and resemble the cnidocils seen by SEM in *Hydra* (Westfall and Enos, 1972; Beams, Kessel and Shih, 1973). Indeed, the distribution of these broad-based cilia parallels the distribution of nematocysts through the polyp epithelia, and discharged nematocyst threads consistently originate at the bases of these structures. The long cnidocil shafts are often entwined around discharged nematocyst threads (Figs. 9, 10) consistent with the observation of Robson (1953) that nematocyst threads rotate as they discharge.

Of special functional significance is the distally directed water currents generated by motile cilia in all regions of the polyp. The speed of these currents appear directly related to the density of cilia in each area. This is most obvious over the heavily ciliated SPC where the current is particularly rapid. Ciliary currents and the increased surface area provided by micromovilli would facilitate the vital diffusion dependent processes of cellular respiration and excretion. The continuous ventilation accomplished by motile cilia, particularly in the gastrocoel, provides a metabolically inexpensive alternative to the periodic ventilation of the organism by body muscle contraction.

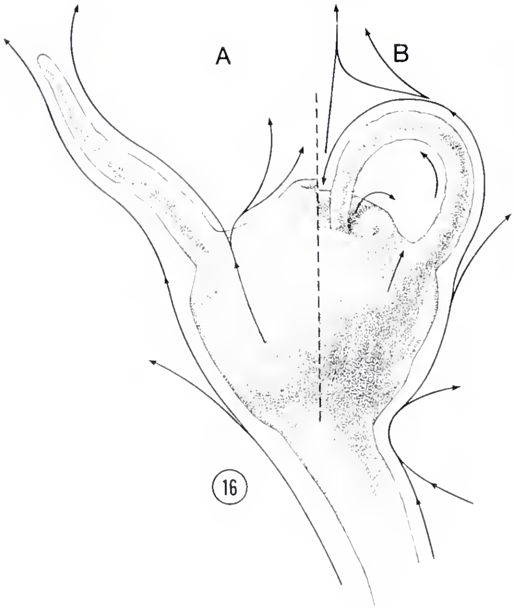
As relatively non-motile, estuarine animals, *Chrysaora* polyps are exposed to a constant shower of silt and fine particulate matter brought in by rivers and tributaries. Polyps rapidly succumb when covered by silt and mud (D. Cargo, Cheaspeake Biological Laboratory, Oxford, Maryland, personal communication) and, hence, distally directed ciliary currents would prevent the accumulation of material in the vicinity of the polyp. In addition, such currents could discourage the settling of larvae which compete with the polyp for available space. In contrast, pond-dwelling, freshwater *Hydra* are sparsely ciliated (Beams, Kessel and Shih, 1973).

The well developed septae of *Chrysaora* polyps provide an increased surface area over which digestive processes occur. As *Chrysaora* often captures and ingests relatively large and active prey such as fish fry and crustaceans, the nematocysts of this region may serve to further sting and immobilize such organisms. Although Chapman (1966) feels that the septae serve to accomodate the cord muscles used for polyp contraction, our observations suggest significant, additional roles. Cells of the SPC actively phagocytize small particles from the gastrocoel as demonstrated by the ingestion of carmine and thus it is likely that they ingest food particles as well. Phagocytosis or lysis of bacteria by digestive enzymes may

---

FIGURE 14. Edge of gastric septum illustrating a large dome-shaped structure surrounded by microvilli. Numerous cnidocils are present; their broad bases consisting of fused microvilli of varying length; 10 μm scale marker.

FIGURE 15. Surface of gastrodermis between the gastric septae. Most cells possess a single cilium and are covered by long microvilli. Microvilli are far more abundant on these absorptive cells than anywhere else on the polyp; 10 μm scale marker.



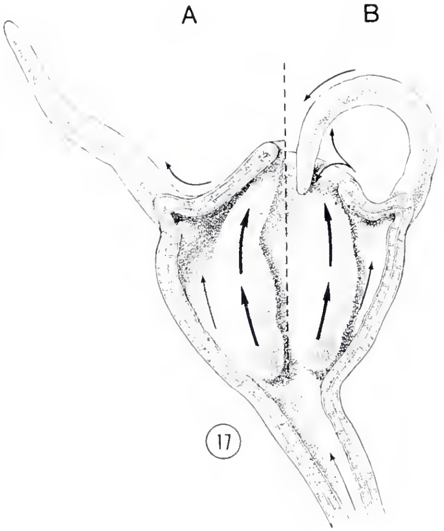


FIGURE 16. Water currents generated by epidermal cilia in non-feeding (A) and feeding (B) polyps.

FIGURE 17. Water currents generated by the gastrodermal cilia in non-feeding (A) and feeding (B) polyps. Currents are particularly rapid along the septal edge.

account for their relative absence on the gastrodermal surface in comparison to the epidermis.

Ciliary currents within the gastrocoel would aid extracellular digestion by mixing ingested food with secreted enzymes. These currents would also increase the number of particle contacts with phagocytic cells. After digestion is complete, the distally directed currents then serve to expel debris and undigested material from the gastrocoel through the open mouth.

The fact that the direction of ciliary currents is unaffected by feeding activators suggests that these currents play little, if any, role in food acquisition. In contrast, other cnidarians such as the sea anemone *Metridium* (Parker and Marks, 1928) demonstrate reversal of ciliary currents which aid in carrying food particles to the mouth.

The authors are grateful to Susan Hester for technical assistance, Harry Schaefer for the photographs and Trudy Nicholson for the drawings.

SUMMARY

A survey of the epidermal and gastrodermal surfaces of the scyphopolyp *Chrysaora quinquecirrha* by scanning electron microscopy reveals that all epithelia

are ciliated to varying degrees. Cilia are surrounded at their bases by microvilli which vary in configuration. These microvillar configurations are characteristic in different areas of the polyp. Cilia with broad conical bases, corresponding closely in distribution with nematocysts, are thought to represent cnidocils.

Cilia on the epidermal and gastrodermal surfaces are responsible for the generation of a continuous current which flows in an aboral to oral direction in both feeding and non-feeding conditions. The strongest currents are observed along the free edges of the gastric septae, which are the regions of heaviest ciliation in the polyp. Externally, such currents aid in the removal of silt and debris in relatively sessile polyp and serve to circulate gastrovascular fluid and to remove undigested materials.

This work was supported by a grant ES00854 from the National Institutes of Health to the senor author.

LITERATURE CITED

Beams, H. W., R. G. Kessel, and C. Y. Shih, 1973. The surface features of *Hydra* as revealed by scanning electron microscopy. *Trans. Amer. Microscop. Soc.*, **92**: 161–175.

Burnett, A. L., and N. A. Diehl, 1964. The nervous system of *Hydra*. I. Types, distribution, and origin of nerve elements. *J. Exp. Zool.*, **157**: 217–226.

Chapman, D. M., 1966. Evolution of the scyphistoma. *Symp. Zool. Soc. London*, **16**: 51–75.

Chapman, G. B., and L. G. Tilney, 1959a. Cytological studies of the nematocysts of *Hydra*. I. Desmonemes, isorhizas, cnidocils and supporting structures. *J. Biophys. Biochem. Cytol.*, **5**: 69–78.

Chapman, G. B., and L. G. Tilney, 1959b. Cytological studies of the nematocysts of *Hydra*. II. The stenoteles. *J. Biophys. Biochem. Cytol.*, **5**: 79–84.

Eakin, R. M., and J. A. Westfall, 1962.. Fine structure of photoreceptors in the hydromedusan, *Polyorchis penicillatus*. *Proc. Nat. Acad. Sci.*, **48**: 826–833.

Horridge, G. A., 1965. Non-motile sensory cilia and neuromuscular junctions in a ctenophore independent effector organ. *Proc. Roy. Soc. London Series*, **B162**: 333–350.

Horridge, G. A., 1969. Statocysts of medusae and evolution of stereocilia. *Tissue and Cell*, **1**: 341–353.

Horridge, G. A., and P. S. Boulton, 1967. Prey detection by Chaetognatha via a vibration sense. *Proc. R. Soc. London Series*, **B168**: 413–419.

Jha, R. K., and G. O. Mackie, 1967. The recognition, distribution and ultrastructure of Hydrozoan nerve nets. *J. Morphol.*, **123**: 43–62.

Josephson, R. K., 1961. The response of a hydroid to weak water-borne disturbances. *J. Exp. Biol.*, **38**: 17–27.

Lentz, T. L., 1966. *The Cell Biology of Hydra.* North-Holland Pub. Co., Amsterdam, 199 pp.

Lentz, T. L., and R. J. Barrnett, 1965. Fine structure of the nervous system of hydra. *Amer. Zool.*, **5**: 341–356.

Loeb, M. J., and R. S. Blanquet, 1973. Feeding behavior in polyps of the Chesapeake Bay sea nettle, *Chrysaora quinquecirrha* (Desor, 1848). *Biol. Bull.*, **145**: 150–158.

Mackie, G. O., 1960. Studies on *Physalia physalis* L. Part 2: Behavior and Histology. *Discovery Reports*, **30**: 369–408.

McConnell, C. H., 1932. The development of the ectodermal nerve net in the buds of Hydra. *Quart. J. Microscop. Sci.* **75**: 495–509.

Parker, G. H., and A. P. Marks, 1928. Ciliary reversal in the sea anemone, *Metridium*. *J. Exp. Zool.*, **52**: 1–6.

Robson, E. A., 1953. Nematocysts of *Corynactis:* the activity of the filament during discharge. *Quart J. Microscop. Sci.*, **94**: 229–235.

Tcheou, T. C., 1930. Le cycle evolutif du scyphistome de *Chrysaora. Trav. Sta. Biol. Roscoff*, **8**: 1–179.

WEILL, R., 1930. Essai d'une classification des nematocystes des cnidaires. *Bull. Biol. Fr. Belg.*, **64**: 141–156.

WEILL, R., 1934. Contributions a l'etude des cnidaires et de leurs nematocysts. *Trav. Sta. Zool. Wimereux,* **10**: 1–347.

WESTFALL, J. A., 1965. Nematocysts of the sea anemone, *Metridium. Amer. Zool.,* **5**: 377–393.

WESTFALL, J. A., 1970. The nematocyst complex in a Hydromedusan, *Gonionemus vertans. Z. F. Zellforsch Mikrosk. Anat.*, **110**: 457–470.

WESTFALL, J. A., AND P. D. ENOS, 1972. Scanning and electron microscopy of the isolated cells of *Hydra littoralis.* 30th Ann. Proc. Electron Micros. Soc. Amer., pp. 160–161. Los Angeles.

Reference: *Biol. Bull.,* **148**: 193–198. (April, 1975)

THE BIOLOGY OF THE LEECH *MYZOBDELLA LUGUBRIS* INFESTING BLUE CRABS AND CATFISH

BRUCE A. DANIELS [1] AND ROY T. SAWYER

Department of Biology and Grice Marine Biological Laboratory, College of Charleston, Charleston, South Carolina 29401

A cyclic seasonal abundance for fish leeches has been reported by Gibson and Tong (1969) and Sawyer and Hammond (1973). Fish leeches leave their hosts to deposit cocoons on rocks (Becker and Katz, 1965), oyster clumps, and other suitable surfaces (Sawyer and Hammond, 1973). From the present work it appears that the piscicolid *Myzobdella lugubris* is parasitic on a piscine host for most of the year and then leaves the fish and deposits cocoons on decapods. This is the first record of a fish leech utilizing both a fish and an arthropod in its life history.

Myzobdella lugubris Leidy 1851 and *Illinobdella moorei* Meyer 1940 have until recently been separated primarily on the basis of salinity and host. *Myzobdella lugubris* was known as a marine leech commensal on crustaceans (Hutton and Sogandares-Bernal, 1959; Pearse; 1936; Sawyer, 1967; Wurtz and Roback, 1955), and *Illinobdella moorei* as a freshwater leech parasitic on fishes (Hoffman, 1967). Sawyer, Lawler and Overstreet (1974) have recently synonymized these two forms with *M. lugubris* taking precedence. The ecological data presented in this paper supports such a synonymy.

MATERIALS AND METHODS

To establish the seasonal abundance of *M. lugubris,* a population was examined at monthly intervals from May, 1972 through April, 1973 in the middle reaches of the Ashley River, Dorchester county, South Carolina (32°55.5′N; 80°08.0′W). Here *M. lugubris* was parasitic in large numbers on the common white catfish, *Ictalurus catus* (Linnaeus). The Ashley River is tidal along most of its length (salinity from 0 to 14‰). The temperature varied from 4° C in January to 29° C in July. The river was exceptionally turbid with a typical Secchi disc reading of 75 cm (range 20 to 125 cm). The bottom and banks of the river consisted primarily of sand and *Spartina* detritus. Very few stones or rocks were present.

The numbers of catfish collected during each monthly sample ranged from 39 to 141. All catfish were captured with a 4.5 m trynet with 0.5 cm square mesh.

---

[1] Present address: Department of Parasitology, School of Hygiene, University of Toronto, Toronto, Canada

Hosts were isolated in plastic bags and placed on ice. In the laboratory the numbers and positions of the leeches on each fish were recorded. Leeches not kept for biological observations were anesthetized by slowly adding small amounts of 90% ethanol and then preserved in 10% formalin.

Specimens and cocoons from this study are deposited in the U. S. National Museum (USNM 49962).

RESULTS

Of the 868 catfish examined 70% were infested with leeches. The average number of leeches per catfish was 2.7 with 1.2 (44%) on the pectoral fins, 0.4 (15%) on the pelvic fins, 0.4 (15%) in the mouth, and the remaining 0.7 (26%) distributed over the rest of the body. The seasonal abundance of *M. lugubris* can be divided into three phases: May through September, during which the population remained relatively stable; October through January, when recruitment to the population occurred; and February through April, when the population underwent a decline (Table I).

TABLE I

Monthly infestation of Myzobdella lugubris *on* I. catus.

Date	Temperature °C	Number of fish examined	Number of fish infested	Number of leeches/fish	
				Range	Mean ± 2 S.E.
V/24/72	22.2	67	33	0–12	1.1 ± 0.2
VI/30/72	26.4	77	28	0–6	0.7 ± 0.2
VII/23/72	29.7	42	0		
VIII/8/72	28.2	45	20	0–2	0.7 ± 0.1
IX/8/72	25.6	141	71	0–4	0.8 ± 0.1
X/7/72	22.0	89	72	0–10	2.3 ± 0.2
X/22/72	17.5	90	78	0–11	3.0 ± 0.3
XI/7/72	18.5	72	68	0–14	3.9 ± 0.3
XII/9/72	13.7	72	58	0–18	3.8 ± 0.4
I/13/73	4.5	66	65	0–48	11.4 ± 1.4
III/18/73	16.9	63	50	0–9	2.4 ± 0.3
IV/21/73	22.6	39	28	0–10	2.4 ± 0.4

Two apparent anomalies existed in the relative abundance of leeches on different parts of the catfish. On 22 October 44% of the leeches were found in the mouth compared with less than 15% for the rest of the year. In January and March 24% and 31% respectively of the leeches occurred on the barbels compared with less than 5% for the rest of the year.

The seasonality was further corroborated by a strong negative correlation between water temperature and the average number of leeches per catfish (r = −0.92) as well as between the temperature and the catfish infestation rate (r = −0.81).

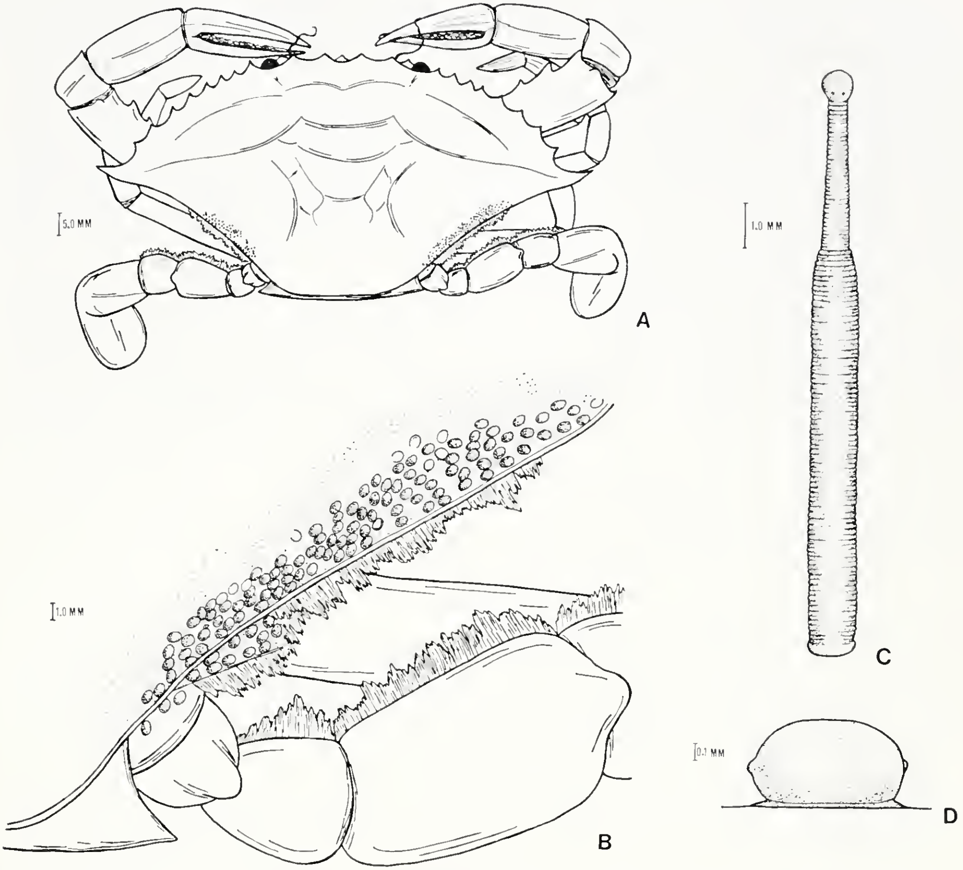


FIGURE 1. Cocoons of *Myzobdella lugubris* on the carapace of Callinectes sapidus: A, *Callinectes sapidus* with cocoons on the postero-lateral regions; B, enlargement of the same postero-lateral region; C, *Myzobdella lugubris*, dorsal view; D, cocoon of *Myzobdella lugubris*. All were drawn from specimens by Ann F. Flowers.

For most of the year the leeches were randomly distributed on the fish population (the variance equal to the mean) but in January the leech distribution was overdispersed (mean much greater than variance).

Throughout the year, especially in October and November, leeches were encountered on the carapace of the blue crab. Upon close examination it became evident that the crab carapace normally served as a site for the deposition of the leech cocoons (Fig. 1).

In a sample of 28 crabs collected on 7 October 1972 in the lower reaches of the Ashley River, 18 crabs with an average of 118 cocoons (range 1–294) had a total of 2123 cocoons. The cocoons were deposited on the postero-lateral regions of the carapace, equally distributed dorsally and ventrally and without regard to sex. Some of the crabs had engorged leeches the guts of which contained nucleated erythrocytes.

Based on one year's data it is suggested that the population of *M. lugubris* on *Ictalurus catus* in the Ashley River undergoes an annual cycle. The number of leeches on the catfish declines in the spring, and increases in the fall. Associated with this fall increase is an increase in the precentage of the population occurring in the mouth. It is apparently here that the leeches become engorged with blood before leaving the catfish to deposit cocoons.

The peak of cocoon deposition occurs during the summer months. However, crabs with cocoons were found in the Ashley River from the last of March until November. No crabs were found in the area sampled during the rest of the year. In the laboratory cocoons hatch in 11–39 days at 23–25° C (Sawyer *et al.,* 1974). It is not known, however, how long it takes the cocoons to hatch at other temperatures. Shortly after the young leeches attach to their host they take their first blood meal and this is followed by a period of rapid growth. A gradual decline in the leech population occurred between January and July.

DISCUSSION

Myzobdella lugubris is the first documented case of a piscicolid leech whose life cycle may normally involve two "hosts," one fish and one crustacean. Similar leeches, *e.g., Crangonobdella murmanica, Glyptonotobdella antarctica,* and *Hemibdella soleae,* are known only from crustacean or fish hosts, never from both (Selensky, 1914, 1915; Llewellyn, 1965; Sawyer and White, 1969). Only three other instances have been fully documented in which piscicolids use arthropods as sites for cocoon deposition. *Crangonobdella murmanica* deposits cocoons on the shrimp *Sclerocrangon boreas* (see Selensky, 1914, 1915; MacGinitie, 1955), *Johanssonia pantopodum* deposits cocoons on the pycnogonid *Nymphon stromi* (see Selensky, 1914, 1915), and *Notostomobdella cyclostoma* deposits cocoons on the crab *Paralithodes camtschaticus* (see Moore and Meyer, 1951). About ten other leeches have been reported on arthropods by various authors, and in more than one case the leeches were thought to parasitize the hosts (Selensky, 1914, 1915; Meyer and Barden, 1955; Sawyer and White, 1969). In spite of these brief reports to the contrary, a case for a piscicolid feeding on an arthropod has never been unequivocally documented. It is more likely that leeches employ arthropods only for locomotion and/or cocoon deposition.

Myzobdella lugubris (= *Illinobdella moorei*) has been found in fresh and brackish water over most of the United States and reported from over two dozen fish species with what appears to be little host preference (Sawyer *et al.*, 1974). Little biological work has been done on the freshwater form, and its life history where crabs and shrimp are absent is not certain. Whether freshwater *Myzobdella* *lugubris* normally utilize stones or other abiotic substrates for cocoon deposition is yet to be answered.

Our special thanks go to Drs. N. A. Chamberlain and H. W. Freeman for supplying facilities and other assistance; to Drs. M. C. Meyer and R. J. G. Lester for critically reviewing the manuscript; and to A. F. Flowers for the illustrations. We also give our grateful thanks to the following people for assistance in collecting leeches: J. F. McKinney, W. A. Roumillat, J. W. Tucker, and J. L. Vilagos of the College of Charleston, and D. L. Hammond, J. S. Hopkins, and B. W. Stender of the S. C. Marine Resources Division. The work contributed by R. T. Sawyer is the result of research sponsored by NOAA Office of Sea Grant, Department of Commerce, under Grant #NG-33-72.

SUMMARY

1. The life history of *Myzobdella lugubris* is described based upon a quantitative study of the leech from white catfish, *Ictalurus catus,* and blue crabs, *Callinectes sapidus,* from the estuaries of Charleston, South Carolina.

2. The leech displayed seasonality on the fish which inversely correlated with water temperature.

3. The greatest increase in the leech population occurred during December.

LITERATURE CITED

Becker, C. D., and M. Katz, 1965. Distribution, ecology and biology of the salmonid leech *Piscicola salmositica* (Rhynchobdellae: Piscicolidae). *J. Fish. Res. Bd. Canada,* **22(5)**: 1175–1195.

Gibson, R. N., and L. J. Tong, 1969. Observations on the biology of the marine leech *Oceanobdella blennii. J. Mar. Biol. Ass. U. K.,* **49**: 433–438.

Hoffman, G. L., 1967. *Parasites of North American Freshwater Fishes.* University of California Press, Los Angeles, 486 p.

Hutton, R., and F. Sogandares-Bernal, 1959. Notes on the distribution of the leech, *Myzobdella lugubris* Leidy and its association with mortality of the blue crab, *Callinectes sapidus* Rathbun. *J. Parasitol.,* **45**: 384, 404.

Llewellyn, L. C., 1965. Some aspects of the biology of the marine leech *Hemibdella soleae. Proc. Zool. Soc. London,* **145**: 509–528.

MacGinitie, G. E., 1955. Distribution and ecology of the marine invertebrates of Point Barrow, Alaska. *Smith. Misc. Coll.,* **128(9)**: 1–201.

Meyer, M. C., and A. A. Barden, Jr., 1955. Leeches symbiotic on Arthropoda, especially decapod Crustacea. *Wasmann J. Bio.,* **13**: 297–311.

Moore, J. P., and M. C. Meyer, 1951. Leeches (Hirudinea) from Alaska and adjacent waters. *Wasmann J. Bio.,* **9**: 11–77.

Pearse, A. S., 1936. Estuarine animals at Beaufort, North Carolina. *J. Elisha Mitchell Sci. Soc.,* **52**: 174–222.

Sawyer, R. T., 1967. The leeches of Louisiana, with notes on some North American species (Hirudinea, Annelida). *Proc. Louisiana Acad. Sci.,* **30**: 32–38.

Sawyer, R. T., and D. L. Hammond, 1973. Observations on the marine leech *Calliobdella carolinensis* (Hirudinea: Piscicolidae), epizootic on the atlantic menhaden. *Biol. Bull.,* **145**: 373–388.

SAWYER, R. T., A. LAWLER, AND R. OVERSTREET, 1975. The marine leeches of the eastern United States and the Gulf of Mexico with a key to the species. *J. Nat. Hist.,* in press.

SAWYER, R. T., AND M. G. WHITE, 1969. A new genus and species of marine leech, *Glyptonotobdella antaractica,* from an Antarctic isopod. *Brit. Ant. Surv. Bull.,* **22**: 1–14.

SELENSKY, W. D., 1914. Über einege auf Arthropoden schmarotzende Ichthyobdelliden. *Zoo. Anz.,* **44**: 270–282.

SELENSKY, W. D., 1915. Études morphologiques et systématiques sur les Hirudinées. I. L'organisation des Ichthyobdellides (original in Russian). Petrograd: 1–252. (English translation by J. P. Moore and M. C. Meyer).

WURTZ, C. B., AND S. S. ROBACK, 1955. The invertebrate fauna of some Gulf Coast rivers. *Proc. Acad. Natur. Sci. Philadelphia,* **107**: 167–206.

Reference: *Biol. Bull.*, **148**: 199–218. (April, 1975)

REPRODUCTION OF THE EXTERNALLY BROODING SEA ANEMONE *EPIACTIS PROLIFERA* VERRILL, 1869[1]

DAPHNE FAUTIN DUNN[2]

Department of Zoology, University of California, Berkeley, California, and University of California Bodega Marine Laboratory, Bodega Bay, California

Brooding in marine invertebrates may be defined as the retention of offspring by a parent through the embryonic stages usually passed in the plankton, thereby shortening or entirely eliminating the dispersal stage. Among sea anemones external brooding, in which the young are attached to or enfolded by the exterior surface of the parent, is an intriguing and uncommon phenomenon. Carlgren's (1949) catalog of actinians lists only 16 externally brooding species of an approximate world-wide total of 800. These species, unlike internally brooding anemones [in which ". . . the young develop, until after metamorphosis, within the parent's body" (Stephenson, 1928, p. 92)], are confined to polar and temperate seas, and at least five of them are entirely subtidal.

Epiactis prolifera, an actinian that broods its young on its lower column (Fig. 1), is "locally abundant" in the lower littoral zone in rocky parts of the Pacific coast of North America from Puget Sound to La Jolla (Ricketts and Calvin, 1968), thus providing a rare opportunity for research on this poorly understood mode of reproduction. Although it is the best known species in a genus containing 16 species (Carlgren, 1949), *E. prolifera* has been the subject of little detailed study. The major references to it are given by Hand (1955), who also provides the most complete morphological description of the animal.

Uchida (1934) and Uchida and Iwata (1954) discuss the anatomy and development of a Japanese actinian identified as *Epiactis prolifera,* but Uchida (personal communication) now believes it to be *Epiactis japonica.* The study by Uchida and Iwata (1954) is the only published account of the development of an externally brooding sea anemone. The literature contains speculative statements about the reproductive biology of *E. prolifera* (*e.g.,* Bovard and Osterud, 1918; Johnson and Snook, 1927; MacGinitie and MacGinitie, 1968; Ricketts and Calvin, 1968), but none is substantiated by published evidence. This study was done to enhance our knowledge of this fascinating species, and to extend our understanding of external brooding in marine invertebrates.

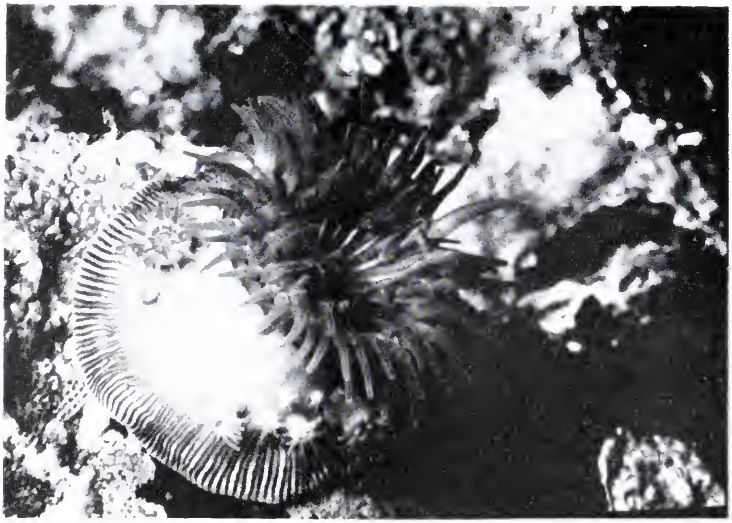
MATERIALS AND METHODS

During the period April 1970 through March 1972, 290 specimens of *Epiactis prolifera* were collected from the rocky open coast northwest of the University of

---

[1] This work was completed in partial fulfillment of the requirements for the Ph.D. degree in the Department of Zoology, University of California, Berkeley, and was supported by an NSF Traineeship.

[2] Present address: School of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia.



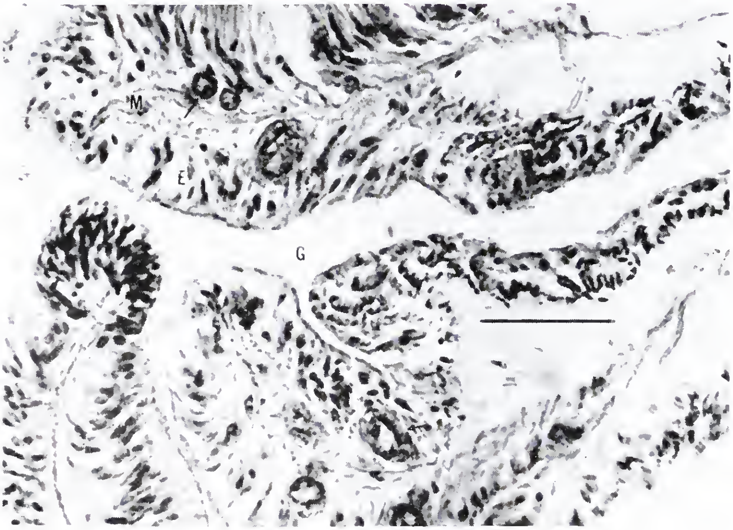


Figure 1. Expanded brooding *Epiactis prolifera*. Juveniles, which are attached low on the parent's column, are of various sizes. (Photograph by Robert Ames.)

Figure 2. Section showing oogonia (distinguishable by their large nuclei) in the endoderm beside the mesoglea of the mesenteries. Of the several oogonia visible, two are indicated by arrows; scale = 40 μ. Labels indicate: M = mesoglea; E = endoderm; EC = ectoderm; G = gastrovascular cavity; O = oocyte; T = tubular channel of the trophonema.

California Bodega Marine Laboratory, Bodega Head, Sonoma County, California. In this locality *E. prolifera* generally occurs below the zero tide level although occasional individuals are found as high as a meter above datum. Thus observations and collections were confined to the monthly periods of extreme low ("spring") tides, except when stormy weather or insufficiently low water prevented access to the animals altogether, and around the times of the spring and autumn equinoxes when the lower intertidal was exposed fortnightly.

Twenty-four collections of adult specimens of *E. prolifera* were made. (I term animals being brooded "juveniles" or "young" while non-brooded individuals are considered adults.) An effort was made to sample animals of a variety of sizes and colors from various sites and tidal levels, and with various numbers of young. Before an animal was removed from the substrate, its color, approximate vertical level, substrate, basal diameter, number of nearby specimens of *E. prolifera,* and number and size class of adherent young were recorded. To standardize conditions for measurement and color determination, each animal was tapped gently and a diving light was shone on it. The light also helped to reveal young. The edge of the brood groove was depressed when necessary to expose hidden juveniles. After prying the animals from the substrate (they come free quite easily with little or no damage), each was put in a plastic bag with an identifying tag.

Prior to fixation, which was done within 24 hours of collection, the anemones were relaxed with saturated chloral hydrate solution (four drops per 100 ml of sea water usually sufficed to make them unresponsive within an hour). The 9:11 menthol:chloral hydrate solution used by Chia and Rostron (1970) proved less satisfactory.

Several fixatives were tried. Bouin's fluid often penetrated *Epiactis* tissues incompletely and unequally, and, more seriously, caused the young anemones to fall off the adult. Zenker's fluid, and sea water formalin both preserved the young on the parent. Helly's fluid was also satisfactory. The approximate degree of shrinkage for each fixative was determined by comparing the basal diameter as measured prior to collection with the basal diameter of the anemone in the paraffin block prior to sectioning. Fixed diameter was 53% of live diameter with Zenker's ($N = 47$), 52% with formalin ($N = 47$), 82% with Bouin's ($N = 4$), and 56% with Helly's ($N = 9$). However, since the pedal disc is likely spread more broadly while still attached to the substrate than after collection, the calculated degrees of shrinkage are probably overestimates. It is also unlikely that all structures shrink equally, so shrinkage of 12 ova, the actual diameter of which were about 400 μ, was calculated. Two Zenker's-fixed animals had recently spawned ova adhering to their bodies, the average diameter of the eggs on one being about 250 μ, and on the other about 300 μ. In another Zenker's-fixed *Epiactis,* freshly-spawned ova, averaging about 300 μ in diameter, had been ingested by attached juveniles. Thus the fixed diameters are about 62.5% and 75% of the actual dimensions. In a Bouin's-fixed anemone, eggs being spawned averaged about 250 μ across in section, approximately 62.5% of the actual diameter. Measurements in the following discussion, all made from fixed, sectioned material with an ocular micrometer, therefore represent 60–75% of the actual dimensions.

Routine staining was done with Ehrlich's hematoxylin and eosin Y, or phosphotungstic acid hematoxylin (Bowling, 1967). Trichrome stains were generally unsatisfactory. Histochemical tests included the Feulgen reaction, alcian blue-PAS, mucicarmine and Leach Bismarck brown (McManus and Mowry, 1960), the Wilson-Ezrin stain (Pearse, 1960), and toluidine blue and Mallory's triple connective tissue stain (Humason, 1967).

Microscopy was done with a Leitz Ortholux, and photomicrographs were taken with a Leitz Orthomat camera attachment using Panatomic X film.

RESULTS

Sexual character of Epiactis

Gametes of *Epiactis prolifera* mature in the mesenteries between the retractor muscles and mesenterial filaments, as in other actinians. They occur in the lower half of the animal both above and below the opening of the actinopharynx into the coelenteron. The sexes of *Epiactis* are not separate, as has previously been reported (Cutress, 1949; Hand, 1955), but not all individuals exhibit hermaphroditism. The distribution of gonads (*i.e.,* gamete-bearing mesenteries) in anemones of various sizes, collected during the two years of this study, is shown in Table I.

TABLE I

Distribution of gametes in animals of various sizes

Size class	Pedal disc diameter at the time of collection (mm)	Total N	Number sterile	Number female	Number hermaphroditic
1	up to 5.5	9	9	0	0
2	5.6–8.0	31	14	17	0
3	8.1–10.5	20	3	16	1
4	10.6–13.0	35	3	27	5
5	13.1–15.5	37	2	26	9
6	15.6–18.0	44	0	22	22
7	18.1–20.5	39	0	20	19
8	20.6–23.0	26	0	6	20
9	23.1–25.5	14	0	3	11
10	25.6 and up	14	0	1	13
		269	31	138	100

All animals with basal diameter less than 5.8 mm were without gonads, and none larger than 15.0 mm was sterile. The largest specimen examined was 35.7 mm.

In hermaphroditic individuals, gametes of the two sexes, in all stages of development, may occur in one mesentery. There is no vertical separation of spermatogenic and oogenic tissue. Sperm follicles and female gametes may alternate randomly in a single mesentery. Some mesenteries contain only the latter and, rarely, some have only sperm. Very early stages of both types of gametes often occur in the most peripheral part of the gametogenic area.

Arrangement of gametogenic mesenteries

Primary and secondary mesenteries are always complete and sterile in adults (I found one anomalous fertile secondary mesentery). Normally only mesenteries of the third and fourth orders are gametogenic, but not all of them in a single anemone are fertile. Fifth order mesenteries, which appear only in larger animals and are generally confined to the basal region, are always sterile. Although the mesenteries are usually regularly arranged through the fourth cycle, the 48 pairs of the fifth order are not added simultaneously. I never found an *Epiactis* with more than 96 pairs of mesenteries.

There is no apparent seasonal gonadal cycle, for animals with gametes in all stages of development are found throughout the year, and sexual state correlates with neither color nor intertidal height at the time of collection, but is related to size. However, changes in sexual state do not take place at a particular size in all animals. This may be partly because size is changeable and difficult to determine accurately in actinians. A parameter closely related to size, the number of mesentery pairs, can be more easily measured, is not subject to change over short periods, and correlates well with sexual state.

Very few anemones with less than four complete cycles of mesenteries contained oogonia or oocytes (the minimum number was 64 in a fertile individual). The proportions of fertile animals in size class 2 with fewer than 48 pairs, and with 48 or more pairs are significantly different. Size class 3 exhibits the same phenomenon less strongly. Oogonia and oocytes of these smallest fertile animals were sometimes in third, sometimes in fourth order mesenteries and, in purely female animals of all sizes, the number of fertile mesenteries of these two cycles were proportionately equal.

The smallest animal with sperm had a basal diameter of 10.1 mm. In size classes 4 and 5, the presence of male gametes was highly correlated with the existence of half or more of the fifth cycle of mesenteries. This correlation also held for size class 6, but not as strongly, and disappeared in size class 7. The one individual in size class 10 lacking sperm had as many mesenteries as the other 13 that were hermaphroditic.

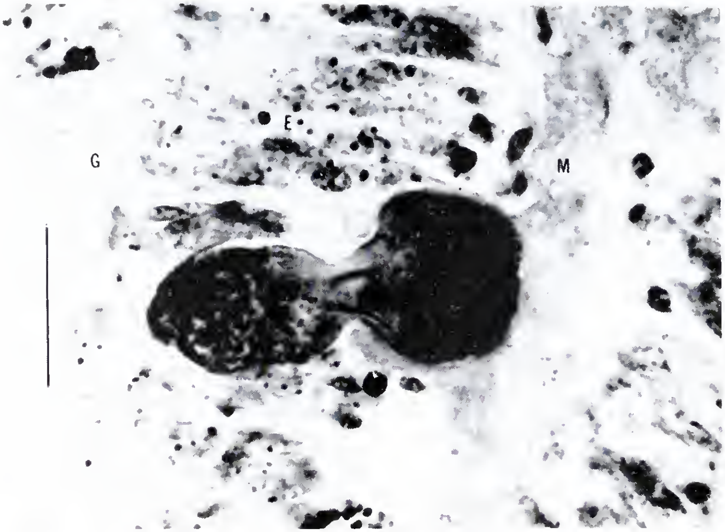
In hermaphrodites, nearly four times as many fourth as third order mesenteries contained ova although there are only twice as many of the former as of the latter, and among the few mesenteries with only sperm, tertiary ones predominated over fourth order ones. There were proportionately equal numbers of third and fourth order hermaphroditic mesenteries.

Fertility and brooding

Many of the animals not brooding at the time of collection were fertile, but all of those brooding young were sexually mature females or hermaphrodites. The smallest fertile adult collected was 5.8 mm in basal diameter, although one precocious adherent juvenile, 2.1 mm in diameter as measured in section, had oogonia and small oocytes. The smallest brooding animal collected was 7.8 mm, from the population of tan *Epiactis* on and around the alga *Cystoseira osmundacea* (Dunn, 1972), but the smallest one with young from coralline algae-encrusted rock was 11.6 mm.

Oogenesis

Anthozoan germ cells arise in the endoderm, presumably from undifferentiated interstitial cells, but mature in the mesoglea of the mesenteries (Hyman, 1940). Figure 2 illustrates the earliest stage at which gonocytes were distinguishable in *E. prolifera,* as cells with relatively huge nuclei. They are small compared to the size



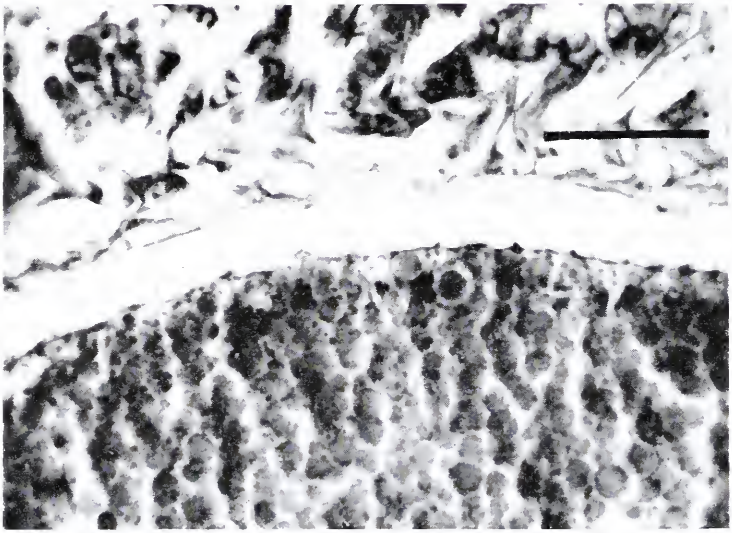


FIGURE 3. Oogonium being thrust from the endoderm into the mesoglea at cytokinesis. The mesoglea appears to bulge out to engulf the egg cell; scale = 20 μ. For labeling, see caption to Figure 2.

FIGURE 4. Section of the edge of a yolky oocyte. Yolk granules are distributed uniformly throughout the cell. Spines on the membrane may be fixation artifacts; scale = 20 μ.

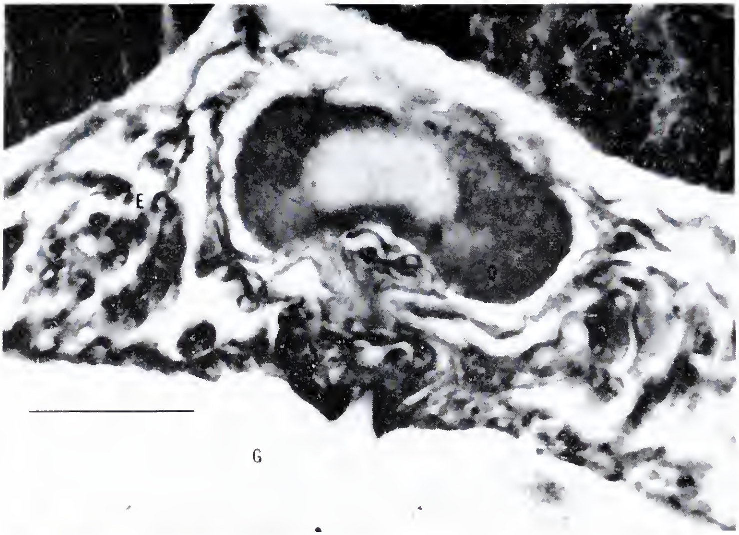
they eventually attain as ova, but are considerably larger than somatic cells. Those in Figure 2 are probably oogonia, although early spermatogonia appear identical. Primary oogonia, ranging in diameter from about 6.0 to 8.5 μ, grow and multiply in the endoderm. Gametes in the endoderm up to 30–35 μ in diameter are secondary oogonia (with nuclei about half the diameter of the cell). These cells move from the endoderm into the mesoglea either by ameboid movement or at division, when one daughter cell is thrust into the mesoglea which appears to bulge to surround the oocyte, leaving the other daughter cell in the endoderm (Fig. 3). Female germ cells usually enter the mesoglea at a diameter of 20–25 μ, and once there cease dividing, indicating that they have become oocytes. As an oocyte grows, the relative diameter of its nucleus changes from half or more that of the entire cell to a quarter or less (the actual size increases somewhat), and concomitantly there is an alteration in the cytoplasm's staining properties reflecting the onset of vitellogenesis. Yolk forms as platelets that are evenly distributed throughout the oocyte, as shown in Figure 4, which also depicts what appear to be spines 1.5–4 μ long on the egg membrane. These are visible from about the time that yolk formation begins, but might be fixation artifacts. The yolk granules concentrate around the germinal vesicle which moves, as the cell grows, from a central to a peripheral position. The germinal vesicle contains a single nucleolus at this stage and is Feulgen-negative. Germinal vesicles of oocytes in the same mesentery and at about the same stage of development exhibit no perferred orientation.

When the oocyte is 30–35 μ in diameter, a bulbous structure becomes apparent between it and the edge of the mesentery (Fig. 5). This soon elongates, forming a hollow tube extending from the edge of the mesentery, penetrating the mesoglea, and indenting the egg cell (Fig. 6). It is 15–20 μ in total diameter, and appears to be identical to a structure first described by Hertwig and Hertwig (1879) from *Sagartia* (now *Calliactis*) *parasitica,* and called by Hertwig (1882) the "filamental apparatus." Hertwig (1882) found it in four other actinian species, and Nyholm (1943) described the same structure from *Cerianthus lloydii,* calling it the "trophonema." I shall use this term for it. In oocytes in which vitellogenesis has begun, the germinal vesicle is located on the side of the oocyte nearest the trophonema. Where it reaches the oocyte, the trophonema flares out, due to the inflated ends of the several cells that comprise the structure and surround its tubular channel. In very large oocytes these cells become considerably flattened (Fig. 7) so that the tubular interior is apparently occluded, and the structure may even seem to disappear.

When full-grown, female gametes break through the thin layer of encapsulating mesoglea and thicker endoderm into the gastrovascular cavity (Fig. 8), whence they travel up the throat and out the mouth.

Spermatogenesis

The first point at which male gametes are unmistakably identifiable is when bundles of 32–64 spermatogonia, each cell averaging 5.5 μ in diameter, are already enveloped by mesoglea. Layering becomes obvious as spermatocytes, average diameter 3.5 μ, come to lie central to the spermatogonia, and as the cells differentiate further, spermatids (1.1–1.3 μ in diameter) occupy the center of the follicle. Finally, in mature follicles, spermatozoa, with apparently conical heads and an average diameter of about 0.7–0.8 μ, fill the center (Fig. 9). Their tails converge toward the edge of the mesentery which will break to release them.



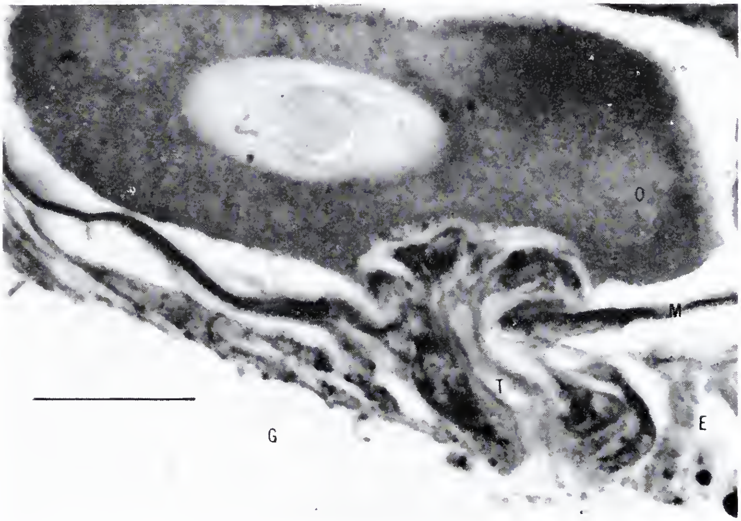
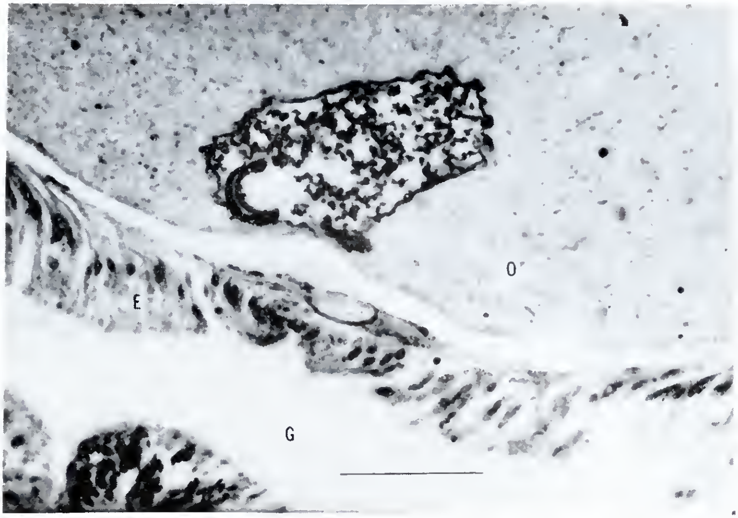


FIGURE 5. Section of a bulbous trophonema between early oocyte and mesentery edge; scale = 20 μ. For labeling, see caption to Figure 2.

FIGURE 6. Indentation of a more mature oocyte by the trophonema. Note that it penetrates the mesoglea and that it has a hollow, tubular interior; scale = 20 μ. For labeling, see caption to Figure 2.

Spawning

On 9 June 1971, I collected two specimens of *Epiactis* from the rocky intertidal for laboratory observation. The next day I noticed in the field that a number of animals were spawning or had done so since the previous day. When I returned



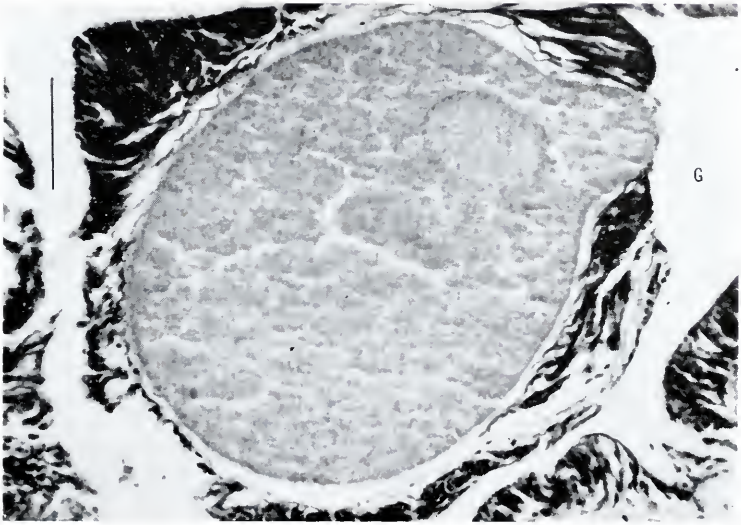
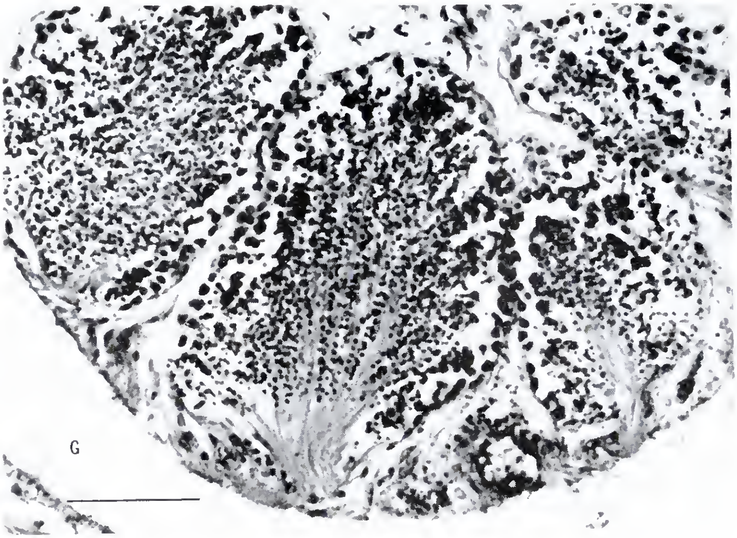


FIGURE 7. Section through a large oocyte with flattened trophonema. The tube appears to have closed by this time; scale = 40 μ. For labeling, see caption to Figure 2.

FIGURE 8. Oocyte breaking through the tissues of the mesentery into the gastrovascular cavity; scale = 50 μ. For labeling, see caption to Figure 2.

to the laboratory, one of the two individuals that I had collected was also spawning. The following day none of the several hundred animals that I carefully examined in the field was spawning, but the second anemone in the laboratory had a number of new spheres attached to its column. I also observed mass spawning in the field and laboratory on 15 and 16 October 1970. In all cases the sexual products expelled were pink or pinkish-orange spheres about 400 μ in diameter. I was un-



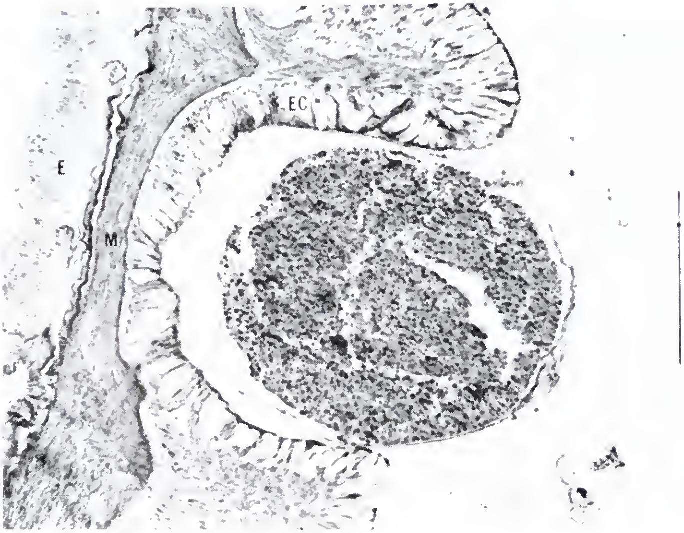


FIGURE 9. Section of a mesentery containing mature sperm follicles. Note the less mature cells peripherally and that the tails of the spermatozoa converge toward the edge of the mesentery; scale = 40 μ. For labeling, see caption to Figure 2.

FIGURE 10. Section of a recently-spawned sphere attached to its parent's body wall. Notice that the yolk has shrunk inside the egg membrane which adheres tightly to the parent ectoderm; scale = 150 μ. For labeling, see caption to Figure 2.

able to determine whether they were ova or zygotes. I never observed the release of sperm.

When I first noticed it in the laboratory, the spawning individual had about 25 spheres, enveloped in a common mass of mucus, on its lips. As the animal (18.7

mm basal diameter and completely covered with water) extended its column and opened and closed its mouth, with concomitant bulging and constriction of the throat and lips, the bundle of spheres twisted, then finally tumbled from the lips to the edge of the oral disc. This process lasted about 10 minutes. More spheres, visible inside the throat when the mouth opened, were gradually expelled a few at a time, ascending not in a siphonoglyph but at the sides of the actinopharynx. At the end of an hour about 50 had been spawned and were resting on one side of the oral disc. These eventually drifted down over the edge of the disc and some of them adhered to the column. During the next several days no development was discernible in either the spheres that had attached or those that had not. The day after spawning the spheres appeared more firmly adherent to the parent ectoderm than they had been originally, but nine days later they had disappeared.

Embryology

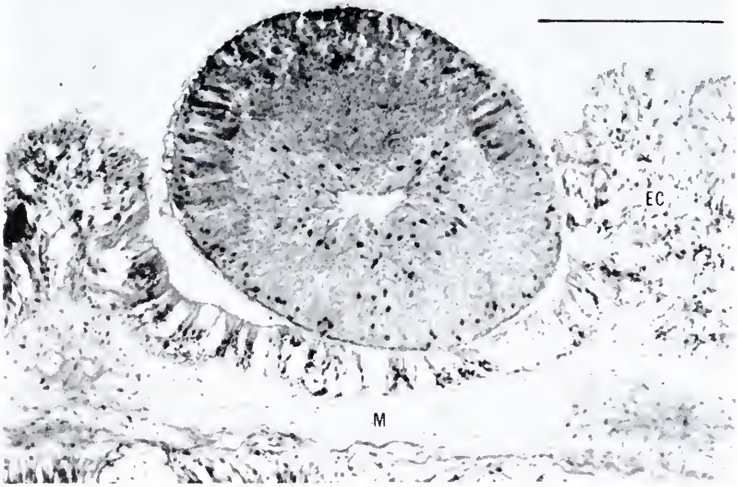
Figure 10 shows a section of an attached, recently spawned sphere. Although the parent body wall does not form an actual brood-pit, the ectoderm is thinned to about half its normal thickness beneath the sphere and thickened around it. The mesoglea is also somewhat thinned, but the endoderm is unaffected.

In an *Epiactis* zygote, the nucleus divides many times before the cytoplasm does, and the daughter nuclei segregate into two distinct layers, foreshadowing the body layers. There is never a blastocoel. Cytokinesis then occurs, resulting in ectodermal cells with nuclei at their distal ends, and endodermal cells with central nuclei (Fig. 11), producing what can only be termed a gastrula. Its high yolk content makes the inner layer considerably thicker than the ectoderm, which is soon devoid of yolk altogether. Mesenteries then form in the yolky interior. The embryo is never ciliated. Tentacle buds arise next, and finally a tiny *Epiactis,* gastrovascular cavity still mostly filled with yolk, is produced (Fig. 12). By the six-mesentery-pair stage most of the yolk is gone, but even before it has been completely absorbed, the juvenile has fully developed nematocysts, spirocysts, and gland cells in its tentacles (Fig. 13). These are functional, and juveniles can capture small organisms (Dunn, 1972, p. 106). The size of the contracted juvenile at this stage is not much greater than that of the zygote.

The ectoderms of parent and juvenile are closely apposed, and the cells of the two are, in material that has not been badly disrupted by histological processing, almost indistinguishable at some points. However, when this intimate relationship is disturbed, a strip of mucoid material is visible between the two animals (Fig. 14). Figure 15 shows an *Epiactis* embryo that has come loose from its parent, with a band of mucus extending from the parent over the embryo. Two types of large and numerous gland cells are present in the parent ectoderm, but there are none in the juvenile.

DISCUSSION

The sexual phenomenon exhibited by *Epiactis prolifera,* wherein the population consists of females and hermaphrodites but no males, is termed gynodioecy (Mather, 1940). In *Epiactis* it resembles protogyny, but there is never complete sexual reversal. Brooding animals are frequently hermaphroditic, as are many sedentary species (Ghiselin, 1969). *E. prolifera* is thus another example of a brooding, sedentary hermaphrodite.



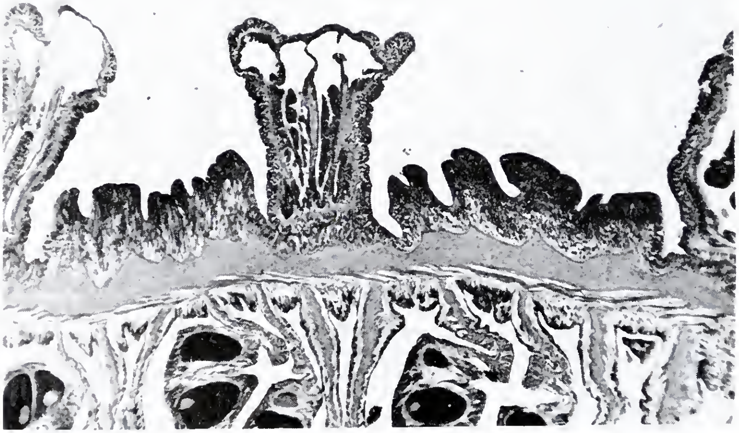
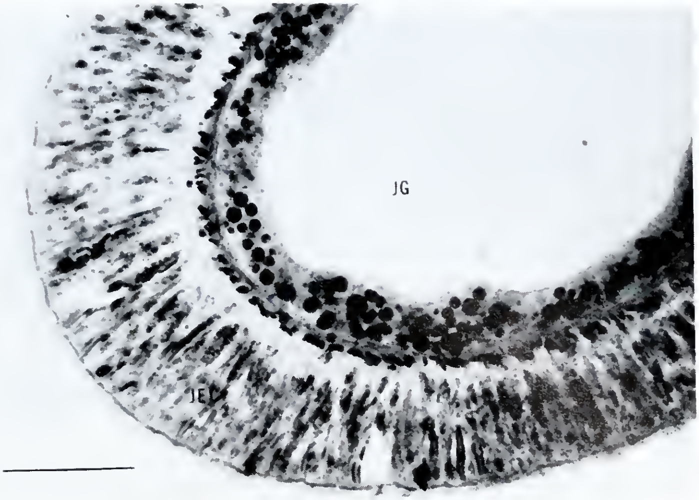


FIGURE 11. Section of an adherent gastrula. Its endoderm is thicker than its ectoderm because of the former's high yolk content; scale = 150 μ. For labeling, see caption to Figure 2.

FIGURE 12. Section through an adult with adherent small juveniles, which are about 2 mm in diameter at this stage.

Earlier reports that *E. prolifera* is dioecious might have resulted from examination of few and/or small individuals. Perhaps, too, gonads were not examined in section so that only larger oocytes, visible to the naked eye, were seen. Even under a dissecting microscope, I had difficulty discerning oogonia, small oocytes and sperm follicles. Large samples of sea anemones of different sizes, collected at various



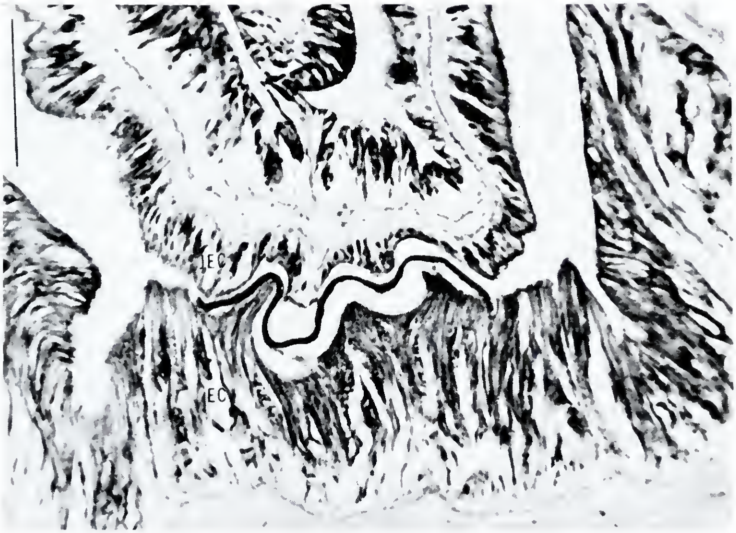


FIGURE 13. Section of a juvenile's tentacle. Spirocysts, nematocysts and gland cells are discernible in the ectoderm, and the endoderm still contains yolk granules; scale = 40 μ. Labels indicate: JG = gastrovascular cavity of the juvenile; JEC = juvenile ectoderm.

FIGURE 14. Strip of mucus between ectoderms of parent and young. The juvenile has become detached; scale = 75 μ. For labeling, see caption to Figures 2 and 13.

times of year must be examined histologically to ascertain a species' sexual character. There may be many more monoecious species of actinians than is commonly believed (Stephenson, 1928, pp. 91–92) because such precautions often have not been observed. Studies of anthozoan reproductive cycles also necessitate histo-

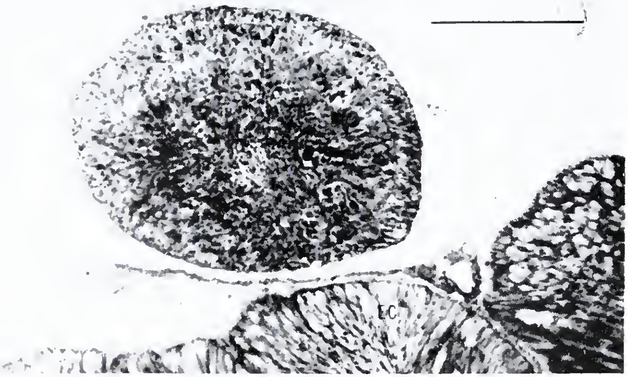


FIGURE 15. *Epiactis* embryo, slightly detached from its parent, with a band of mucus extending over it from the parent; scale = 150 μ. For labeling, see caption to Figure 2.

logical examination. Since sea anemones do not have discrete gonads, I believe that a standard gonad index, as Ford (1964) used in his study of *Anthopleura elegantissima,* can be only approximately correct, at best.

Absence of vertical separation of gametes of the two sexes in hermaphroditic individuals is different from the situation in *Hydra,* where the spermary is above the ovary (Hyman, 1940), and in *Epiactis japonica,* where the situation is reversed (Uchida and Iwata, 1954).

The 12 tertiary pairs of mesenteries, some of which may be fertile, are attached to the oral end of the throat in some larger individuals, so fertile complete mesenteries can occur. Although Hand (1955) denies this, and revises Carlgren's (1949) generic definition to conform to his observations, Verrill's (1899, pp. 377–378) expanded description of *Epiactis prolifera* includes the phrase "12 or more pairs are perfect and fertile." McMurrich (1901) found that all except the first and fifth cycles of mesenteries may be fertile, and that the secondaries may also be complete. These diverse observations reflect the variability of the species, but none accurately describes the situation I found. An emendation of Hand's (1955, p. 39) revision which includes the possibility of perfect and fertile third order mesenteries would read: "At least 12 pairs of mesenteries perfect. . . . Gonads on all the stronger mesenteries, or with the perfect mesenteries *of the first two orders* sterile and the stronger (*including perfect*) mesenteries of the later orders fertile" (italicized words are my emendations).

The regularly hexamerous arrangement of the mesenteries of *Epiactis* suggests that it does not reproduce asexually. Of the 274 animals that I examined in cross-section, only one had three pairs of directive mesenteries, and one had one pair.

Oogonia begin to develop, apparently, at about the time the fourth cycle of mesenteries is completed, regardless of season. Since there are always adults of all sizes, some individuals are completing their fourth cycle of mesenteries at all times of the year.

The 72-mesentery-pair stage also appears critical in the gametogenic physiology of *E. prolifera.* A few individuals seem never to become hermaphrodites and others apparently begin spermatogenesis at all times after the formation of 72 pairs, but the change from female to hermaphrodite in most animals coincides with the formation of half the fifth cycle of mesenteries. Unlike the onset of oogenesis, however, first spermatogenesis may be related to external events. The six smallest hermaphroditic anemones were collected on four different occasions during December and March. It is unlikely that all should have been collected at about the same time of year purely by chance. There is no obvious extrinsic trigger, however, since water temperature in those four months was not equal, nor was it at an extreme, and the animals, each a different color, were from a variety of areas and intertidal heights.

The onset of oogenesis and spermatogenesis may be correlated only weakly with age of an individual. The number of mesenteries increases as the animal grows, but that is not a necessary consequence of increased age since sea anemones grow only if fed, and may even shrink if starved (Chia and Spaulding, 1972).

The data on distribution of gametes in mesenteries suggest that in hermaphrodites mesenteries of the third cycle are somewhat more male and those of the fourth cycle are somewhat more female. This is paradoxical because the tertiary mesenteries are the older ones, but eggs develop before sperm. It is, however, similar to the situation in *Epiactis japonica* (Uchida and Iwata, 1954).

The ovum of *Epiactis,* although yolky, is intermediate in size for a sea anemone (Dawydoff, 1928). The movement of the germinal vesicle to the side of the oocyte during its growth is typical of anthozoans (Chia and Rostron, 1970; Gemmill, 1920; Gohar and Roushdy, 1961; Hill, 1905; McMurrich, 1890; Nyholm, 1959) as well as other cnidarians (Beckwith, 1914; Hargitt, 1906; Hargitt, 1909). The lack of polarized orientation and synchrony of development of female gametes within each mesentery in *E. prolifera* contrasts with the situation in *Actinia equina* (Chia and Rostron, 1970). The ova of some sea anemones float when spawned (Chia and Spaulding, 1972; Gemmill, 1920, 1921; Siebert, 1973), while those of others sink (Gemmill, 1920; Nyholm, 1959). Clearly the negative buoyancy of *Epiactis* ova is absolutely necessary to the species' mode of reproduction.

Spermatogenesis, with its layering of successive stages in the follicles, is typical of most animals. *Epiactis* spermatozoa are about the same size as those of *Actinia equina* (Chia and Rostron, 1970) but considerably smaller than those of *Anthopleura stellula* (Schmidt, 1970).

As the oocytes and sperm follicles grow, the mesenteric mesogleal strand thins around them. Why they should mature within this layer, when they arise in the endoderm and must break out through it to be spawned, has never been explained. One function hypothesized for the mesoglea is storage (Chapman, 1966) and, if this were so, it might be reasonable for growing gametes to localize there where stored nutrients would be readily available. However, the Hertwigs (1879; Hertwig 1882) and Nyholm (1943) believe the trophonema has a nutritive function, and, from a structural standpoint, it does seem admirably suited to deliver nutrients from the gastrovascular cavity to the growing oocyte. Its appearance in *Epiactis* coincides with the onset of vitellogenesis, yolk granules accumulate in the region of its contact with the oocyte, it alters its form at the end of oocyte growth, and there are no nurse cells in *E. prolifera.* These findings strengthen the nutritive hypothesis. Radiotracer studies might be useful in confirming or disproving it.

If this is indeed its function, it might be expected to occur in most anthozoan species, where the growing gametes are separated from the digestive and absorptive surface, but few studies of anthozoan reproduction mention it. It would also leave unexplained the reason for the gametes' migration into the mesoglea to mature.

It has been suggested that sperm might reach an oocyte through the channel of the trophonema. Because of the likelihood of post-spawning fertilization (see below), and because the structure appears so early in the oogenic process and the channel closes or disappears entirely at just about the time the egg cell finishes growing and might be ready to be fertilized, this is unlikely. [Development of the trophonema in *Epiactis* is the same as that described by Nyholm (1943) for *Cerianthus lloydii.*]

When and where reduction division and fertilization (not necessarily in that order) occur in *E. prolifera* are unknown, but I infer that neither happens before spawning because there is no division of the eggs while in the mesenteries, unlike in *Actinia equina* (Chia and Rostron, 1970), and the oocyte shown escaping from the mesentery in Figure 8 still has a large germinal vesicle. All large oocytes in the mesoglea have a conspicuous nucleus, but those in the throat of an animal fixed while spawning have no detectable nucleus, as is true of other species of sea anemones in which fertilization does not occur until after the ova have been spawned (Chia and Spaulding, 1972; Gemmill, 1921; McMurrich, 1890; Nyholm, 1959).

Although gametes in all stages of maturity are present throughout the year, external stimuli are almost certainly required for spawning, since it is at least sometimes an epidemic phenomenon. Spawning, either epidemic or not, occurs during much of the year, for at least 27% of the rocky intertidal population was brooding at any time, and spawning or recently-spawned individuals were found during seven months of the year (February through July, and October) (Dunn, 1972). Thus *E. prolifera* resembles many other temperate species that produce large yolky eggs or brood, in having an extended breeding season (Giese, 1959; Thorson, 1936).

Because it is gynodioecious, there is certainly some outbreeding in *Epiactis,* but larger individuals might be self-fertile since their sperm and ova appear to be ripe simultaneously. This is unlikely, however, for when such hermaphrodites are studied in detail, it is usually found that while self-fertilization may be possible, there are mechanisms to prevent it (*e.g.* Silén, 1966).

The oocytes (or ova?) found in the gullets of juveniles, and the earliest stages attached to parent ectoderm have no visible nucleus, so it is uncertain whether they are fertilized by this time either. Perhaps eggs adhere to parent ectoderm and are fertilized there (maturation divisions may even occur there), but in the event that development does not proceed, the ectoderm releases the sphere. This would be adaptive since it would maximize the productive use of the limited brood area. It might also account for the observation that the oocytes that were spawned in the laboratory and became attached to the parent column had disappeared nine days later. Since the adult was alone in a finger bowl, the spheres were probably unfertilized (unless self-fertilization or parthenogenesis can occur). Or perhaps non-viable oocytes, ova and zygotes simply deteriorate and are washed away.

The finding of freshly-spawned oocytes in the thorats of (sterile) juveniles supports the hypothesis that maximum brood size is limited by the amount of brood space (Dunn, 1972). As eggs spawned by a parent already brooding fairly large young tumble down its column, some may fall onto empty spots of the brood area, keeping it filled, some may fall onto the substrate, but others may fall onto the juveniles' tentacles and provoke a feeding response. They might not be digested by the juveniles, however, since adult specimens of *Epiactis* will ingest other *Epiactis* one-tenth their size, but within several hours will regurgitate them unharmed (Lenhoff, 1965). Should the oocytes in the juveniles' gullets be egested and fall onto unoccupied space in the parental brood area, it is possible that they would develop into juvenile anemones. In fact, since not all eggs that are spawned adhere immediately to the parent ectoderm, this may be a way of reducing loss of them.

It might be hypothesized, by analogy with *Actinia equina* (Chia and Rostron, 1970), that young *Epiactis* are spawned into the plankton, or remain briefly on the parent column before entering the plankton, only later settling on "foster parents." The facts that no *E. prolifera* brooding young was sterile, that all stages of embryogenesis were found among young being brooded, and that parent and juvenile coloration are so similar (Dunn, 1972) make it virtually certain that each animal carries its own offspring.

Dawydoff (1928) states that as many as 32 daughter nuclei may form in anthozoan zygotes before cellularization begins, but it appears that in *Epiactis* the number is greater. Meroblastic cleavage occurs in other anemones, but whereas an ectodermal epithelium forms around a mass of uncleaved yolk in *Actinia bermudensis* (Cary, 1910), *Tealia crassicornis* (Chia and Spaulding, 1972) and *Stomphia didemon* (Siebert, 1973), in *E. prolifera* the endoderm is composed of a few large cells from the onset of cytokinesis. In none of these species does a blastocoel develop.

Johnson and Snook (1927) and MacGinitie and MacGinitie (1968) state that the larvae of *E. prolifera* develop to a considerably advanced stage in the parent enteron, then make their way to the outside where they attach to the parent. They may have been influenced in their incorrect interpretation of the sequence of events by Carlgren (1901), who found no juvenile *Epiactis* without pharynx and mesenteries being brooded, so concluded that none in less developed stages ever occurs on the outside.

Although there are no distinct brood-pits in *E. prolifera*, the ectoderm around each embryo enfolds it, thereby preventing its falling off, but it is unlikely that the ectodermal response is sufficiently rapid to catch a falling sphere as it tumbles down the column. The freshly-spawned spheres probably first adhere in another manner. Mucoprotein secreted by gland cells in the parent ectoderm may be the original cause of adhesion of the spheres. In later stages, mucus covers the embryos, which aids in holding them on the column (Fig. 15), and this is supported by the observation that if a juvenile is pulled off its parent, a band of stringy mucus encircling the brood area becomes apparent. When it is carefully peeled off the adult, some of the smallest juveniles come with it. In juveniles, mucus acts as an adhesive between the ectoderms of parent and offspring (Fig. 14). Uchida and Iwata (1954) believe that mucus is very important in adhesion of young *Epiactis japonica* to the columns of their parents.

Large atrichous nematocysts, heretofore reported in *E. prolifera* only by K. W. England [British Museum (Natural History), personal communication to C.

Hand] occur in the brood area. In the population I studied, they measure 23.1–29.4 × 2.1–4.2 μ (N = 26) in fixed animals, and 24.0–31.7 × 2.9–4.3 μ (N = 20) in fresh smears. I did not find them in small, non-brooding individuals. The restriction of these nematocysts to the lower column of larger adults suggests that they might help to hold the young on the parent column, perhaps being particularly important in initiating adhesion.

It is likely that the parent remains the active partner in maintaining the relationship for its entire duration because even relatively large juveniles have no mucoprotein gland cells nor nematocysts in their columns. When a juvenile finally leaves, it is probably because it has become sufficiently strong to overcome the adhesive forces of the parent or because it is pushed off by growing siblings (Dunn, 1972).

The intimate association between parent and young suggests a nutritive relationship, a possibility considered likely by Verrill (1869) in his original description of *E. prolifera*. However, like other brooders (Thorson, 1950), *Epiactis* produces few large yolky eggs relative to nonbrooding species. Thus the parent has already provided the developing offspring with an energy reserve, and further contribution to its nourishment would appear to be redundant and probably unnecessary (*cf.* Silén, 1945). Although it seems unlikely, experiments using radioactive food could be done to determine whether translocation of nutrients from parent to young does occur. *Epiactis* not only has large yolky eggs, but even tiny juveniles are capable of capturing small prey. They may derive a nutritional advantage from their close proximity to their parents, since they do obtain bits of the food held by their parents or contained in the egestate of the parents (Dunn, 1972). Thus food items which might be too large or strong for them to obtain by themselves are available for sharing by the young. The range in size and variety of potential prey is therefore probably greater for attached young than it would be for isolated animals of the same size.

I thank Dr. Cadet Hand, who first pointed out to me the lack of knowledge about external brooding in actinians and the unusually favorable opportunity for its study in *Epiactis prolifera*, for his help and advice; the faculty, staff and students of the Zoology Department of the University of California/Berkeley and of the Bodega Marine Laboratory for their aid and suggestions, in particular Dr. Ralph I. Smith; the late Dr. J. Ralph Andy, Director of the Hooper Foundation, University of California/San Francisco, for use of facilities in the Foundation; Mr. Stanley Watkins, for excellent instruction in microtechnique; Mr. Charles Fautin, for field assistance; two anonymous reviewers; and, most gratefully, my husband, Fred.

SUMMARY

1. *Epiactis prolifera* is a gynodioecious hermaphrodite, most of the intermediate-size individuals being female and most of the larger ones hermaphroditic. The population studied lacked purely male individuals.

2. Fertile animals may be found not brooding young, but all brooding individuals examined were fertile. Both females and hermaphrodites brood.

3. Gametogenesis proceeds in characteristic fashion, although oogenesis is accompanied by the formation of a trophonema between the oocyte and edge of the mesentery. All stages of both types of gametes were present throughout the year.

4. The zygotic nucleus divides a number of times and the resulting nuclei segregate into two layers before cytokinesis occurs. Mesenteries form while endodermal cells are still yolk-laden, and before it is all absorbed, tentacles with functional nematocysts and gland cells develop. Thus attached juveniles are capable of capturing prey.

5. Mucus and perhaps nematocysts, both of parental origin, are probably responsible for adhesion of the young to the parent's column. Enfolding by the parent ectoderm may also play a role in the early stages.

LITERATURE CITED

Beckwith, C., 1914. The genesis of the plasma-structure in the egg of *Hydractinia echinata. J. Morphol.*, **25**: 189–251.

Bovard, J. F., and H. L. Osterud, 1918. Partial list of the animals yielding embryological material at the Puget Sound Biological Station. *Publ. Puget Sound Biol. Sta.*, **2**: 127–137.

Bowling, M. C., 1967. *Histopathology Laboratory Procedures.* U. S. Department of Health, Education and Welfare, Public Health Service, Public Health Service Publication 1595, 125 pp.

Carlgren, O., 1901. Die Brutflege der Actinarien. *Biol. Centralblatt*, **21**: 468–484.

Carlgren, O., 1949. A survey of the Ptychodactiaria, Corallimorpharia and Actiniaria. *K. Svenska Vetenskap. Handl., Fjärde Serien*, **1**: 1–121.

Cary, L. R., 1910. The formation of germ layers in *Actinia bermudensis* Verr. *Biol. Bull.*, **19**: 339–346.

Chapman, G., 1966. The structure and functions of the mesogloea. *Symp. Zool. Soc. London*, **16**: 147–168.

Chia, F. S., and M. A. Rostron, 1970. Some aspects of the reproductive biology of *Actinia equina* (Cnidaria: Anthozoa). *J. Mar. Biol. Ass. U. K.*, **50**: 253–264.

Chia, F. S., and J. G. Spaulding, 1972. Development and juvenile growth of the sea anemone, *Tealia crassicornis. Biol. Bull.*, **142**: 206–218.

Cutress, C. E., 1949. The Oregon shore anemones (Anthozoa). *M. S. thesis, Oregon State College*, 71 pp.

Dawydoff, C., 1928. *Traité d'embryologie comparée des invertébrés.* Masson et Cie., Paris, 930 pp.

Dunn, D. F., 1972. Natural history of the sea anemone *Epiactis prolifera* Verrill, 1869, with special reference to its reproductive biology. *Ph.D. thesis, University of California, Berkeley*, 187 pp.

Ford, C. E., Jr., 1964. Reproduction in the aggregating sea anemone, *Anthopleura elegantissima. Pac. Sci.*, **18**: 138–145.

Gemmill, J. F., 1920. The development of the sea-anemones *Metridium dianthus* (Ellis) and *Adamsia palliata* (Bohad). *Phil. Trans. Roy. Soc. London, Series* **B209**: 351–375.

Gemmill, J. F., 1921. The development of the sea anemone *Bolocera tuediae* (Johnst.). *Quart. J. Microscop. Sci.*, **65**: 577–587.

Ghiselin, M. T., 1969. The evolution of hermaphroditism among animals. *Quart. Rev. Biol.*, **44**: 189–208.

Giese, A. C., 1959. Comparative physiology: annual reproductive cycles of marine invertebrates. *Ann. Rev. Physiol.*, **21**: 547–576.

Gohar, H. A. F., and H. M. Roushdy, 1961. On the embryology of the Xeniidae (Alcyonaria) (with notes on the extrusion of the larvae). *Publ. Mar. Biol. Sta. Al-Ghardaqa*, **11**: 43–70.

Hand, C., 1955. The sea anemones of central California. Part II. The endomyarian and mesomyarian anemones. *Wasmann J. Biol.*, **13**: 37–99.

HARGITT, C. W., 1906. The organization and early development of the egg of *Clava leptostyla* Ag. *Biol. Bull.,* **10**: 207–232.

HARGITT, G. T., 1909. Maturation, fertilization, and segmentation of *Pennaria tiarella* (Ayres) and *Tubularia crocea* (Ag.). *Bull. Harvard Mus. Comp. Zool.,* **53**: 161–212.

HERTWIG, O., AND R. HERTWIG, 1879. *Die Actinien.* Verlag von Gustav Fischer, Vormals Friedr. Mauke, Jena, 224 pp.

HERTWIG, R., 1882. Report on the Actiniaria dredged by H. M. S. Challenger, during the years 1873–1876. *Rpt. Sci. Res. H. M. S. Challenger, Zoology,* **6**: 1–136.

HILL, M. D., 1905. Notes on the maturation of the ovum of *Alcyonium digitatum. Quart. J. Microscop. Sci.* **49**: 493–505.

HUMASON, G. L., 1967. *Animal Tissue Techniques,* [2nd ed.]. W. H. Freeman and Co., San Francisco and London, 569 pp.

HYMAN, L. H., 1940. *The Invertebrates: Protozoa through Ctenophora.* McGraw-Hill Book Co., New York and London, 726 pp.

JOHNSON, M. E., AND H. J. SNOOK, 1927. *Seashore Animals of the Pacific Coast.* MacMillian Co., New York, 659 pp.

LENHOFF, H. M., 1965. Mechanical stimulation of feeding in *Epiactis prolifera. Nature,* **207**: 1003.

MACGINITIE, G. E., AND N. MACGINITIE, 1968. *Natural History of Marine Animals,* [2nd ed.]. McGraw-Hill Book Co., New York, 523 pp.

McMANUS, J. F. A., AND R. W. MOWRY, 1960. *Staining Methods: Histologic and Histochemical.* Paul B. Hoeber, Inc., New York, 423 pp.

McMURRICH, J. P., 1890. Contributions on the morphology of the Actinozoa. II. On the development of the Hexactiniae. *J. Morphol.,* **4**: 303–330.

McMURRICH, J. P., 1901. Report on the Hexactiniae of the Columbia University expedition to Puget Sound during the summer of 1896. *Ann. N. Y. Acad. Sci.,* **14**: 1–52.

MATHER, K., 1940. Outbreeding and separation of the sexes. *Nature,* **145**: 484–486.

NYHOLM, K. G., 1943. Zur Entwicklung und Entwicklungsbiologie der Ceriantharien und Aktinien. *Zool. Bidr. Uppsala,* **22**: 87–248.

NYHOLM, K. G., 1959. On the development of the primitive actinian *Protanthea simplex,* Carlgren. *Zool. Bidr. Uppsala,* **33**: 69–77.

PEARSE, A. G., 1960. *Histochemistry: Theoretical and Applied,* [2nd ed.]. J. and A. Churchill, Ltd., London, 998 pp.

RICKETTS, E. F., AND J. CALVIN, 1968. *Between Pacific Tides,* [4th ed., revised by J. W. Hedgpeth] Stanford University Press, Stanford, California, 614 pp.

SCHMIDT, H., 1970. *Anthopleura stellula* (Actiniaria, Actiniidae) and its reproduction by transverse fission. *Mar. Biol.,* **5**: 245–255.

SIEBERT, A. E., JR., 1973. A description of the sea anemone *Stomphia didemon* sp. nov. and its development. *Pac. Sci.,* **27**: 363–376.

SILÉN, L., 1945. The main features of the development of the ovum, embryo and ooecium in the ooeciferous Bryozoa Gymnolaemata. *Ark. Zool.,* **35(17)**: 1–34.

SILÉN, L., 1966. On the fertilization problem in the gymnolaematous Bryozoa. *Ophelia,* **3**: 113–140.

STEPHENSON, T. A., 1928. *The British Sea Anemones, Vol. 1.* Ray Society, London, 148 pp.

THORSON, G., 1936. The larval development, growth, and metabolism of Arctic marine bottom invertebrates compared with those of other seas. *Medd. Grønland,* **100(6)**: 1–155.

THORSON, G., 1950. Reproductive and larval ecology of marine bottom invertebrates. *Biol. Rev. Cambridge Phil. Soc.,* **25**: 1–45.

UCHIDA, T., 1934. A brood-caring actinian subject to a wide range of colour variation. *J. Fac. Sci. Hokkaido Imp. Univ., Series 6,* **3**: 17–31.

UCHIDA, T., AND F. IWATA, 1954. On the development of a brood-caring actinian. *J. Fac. Sci. Hokkaido Univ., Series 6,* **12**: 220–224.

VERRILL, A. E., 1869. Notes on Radiata in the museum of Yale College, with descriptions of new genera and species. *Trans. Conn. Acad.,* **1**: 247–596.

VERRILL, A. E., 1899. Descriptions of imperfectly known and new actinians, with critical notes on other species, V. *Amer. J. Sci.,* **157**: 375–380.

Reference: *Biol. Bull.*, **148**: 219–242. (April, 1975)

GONADAL DEVELOPMENT DURING THE ANNUAL REPRODUCTIVE CYCLE OF *COMANTHUS JAPONICA* (ECHINODERMATA: CRINOIDEA)

NICHOLAS D. HOLLAND, JOHN C. GRIMMER AND HIROSHI KUBOTA

Division of Marine Biology, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92037, and Misaki Marine Biological Station of the University of Tokyo, Misaki, Kanagawa-ken, Japan

Echinoderm gonads have been described from periodic histological examinations made throughout the annual reproductive cycle in the following classes: Holothuroidea (Tanaka, 1958); Ophiuroidea (Patent, 1969; Fenaux, 1970, 1972); Asteroidea (Pearse, 1965; Chia, 1968; Crump, 1971); and Echinoidea (Yoshida, 1952; Fuji, 1960; Holland and Giese, 1965; Holland, 1967; Fenaux, 1968; Pearse and Phillips, 1968; Holland and Holland, 1969; Holland and Rommel, 1969; McPherson, 1969; Pearse, 1969a, 1969b, 1970; Gonor, 1973a, 1973b). No comparable study has yet been published for any species of the echinoderm class Crinoidea. Therefore, the present investigation describes gonadal development during the annual reproductive cycle of *Comanthus japonica* (Müller), an unstalked crinoid. The anatomy and histology of the crinoid reproductive system are diagrammed in Figure 3 of Holland (1975).

Materials and Methods

We studied a population of *Comanthus japonica* living on rocks at the entrance of Koaziro Bay, Kanagawa Prefecture, Japan. At each collection we captured 3 to 25 adult crinoids by diving in a few meters of water. The distribution of male, female and unsexable animals was as follows: 14 December 1972 (1 M + 7 F); 1 March 1973 (3 M + 7 F); 7 May 1973 (5 M + 4 F); 1 July 1973 (3 M + 4 F); 4 August 1973 (5 M + 4 F); 18 August 1973 (5 M + 4 F); 2 September 1973 (2 M + 3 F); 15 September 1973 (3 F); 24 September 1973 (8 F); 27 September 1973 (14 F); 30 September 1973 (12 F); 2 October 1973 (3 M + 13 F); 7 October 1973 (4 M + 14 F); 11 October 1973 (13 M + 12 F); 22 October 1973 (5 M + 4 F + 4 U); 8 November 1973 (4 M + 7 U); 24 November 1973 (2 F + 8 U); and 1 February 1974 (4 M + 6 F). For the samples of 15 September 1973 through 2 October 1973, we deliberately collected more females than males; however, for 14 December 1972 and 7 October 1973, the predominance of females over males was presumably due to random sampling error. The collected animals had 28 to 42 arms each and had disc diameters from 2.4 to 3.7 cm; crinoids in this size range had presumably been through at least one annual reproductive cycle before our study commenced.

On the day of collection, several genital pinnules were removed from each animal about a fifth of the way between the arm base and the arm tip; they were fixed at once in sea water-Bouin's fluid. The fixation of 2 October 1973 was several hours before spawning took place. After fixation, genital pinnules were dehydrated in ethanol, cleared in xylene, embedded in paraffin and sectioned at 7 μ. Some sections were stained with alcian blue at pH 3, azure A, mercuric bromphenol blue or periodic acid-Schiff (PAS) under conditions stated on page 284 of Holland and Nimitz (1964); before staining with PAS, glycogen was removed with diastase (Pearse, 1960). Other sections were stained with haematoxylin and eosin, with haematoxylin and PAS, or with alcian blue and PAS.

For each gonad, the volumes of several cell populations were determined by a method not previously described. A histological cross section was cut a fourth of the way between the base and the tip of the gonad. The section was stained with haematoxylin and PAS, and its image was projected by means of a *camera lucida* onto a sheet of aluminium foil at a magnification of 88×. For each ovary, the outlines of the post-pachytene oocytes (without extracellular jelly, if present) and the lumen were outlined on the foil. Regions outlined for each testis were the spermatogonia, the spermatocytes together with spermatids and the spermatozoa. Each aluminium foil image was cut out and weighed to the nearest tenth of a milligram. These weights were then divided by the weight of an aluminium foil square 88 mm on a side in order to convert weight to area. The gonad (which actually tapers gently along the distal fourth of its length) was assumed to be a solid cylinder with a constant diameter all along its length. Gonad length (measured from dissections of fixed pinnules adjacent to those prepared for histology) was multiplied by area to yield the volume of each region in mm^3 per gonad.

Fluctuations in the size-frequency structure of the post-pachytene oocyte population were studied by the frequency polygon method of Pearse (1965, p. 53). In histological sections of each ovary, the diameters of the first fifty oocytes encountered in nucleolar section were measured with an occular micrometer. Since most oocytes were somewhat elliptical in outline, the diameter was calculated by adding the long and short axes of the ellipse and dividing by two. When an oocyte was dented (as in Fig. 15), the diameter was measured from the ridge crests on one side of the cell to the ridge bases on the opposite side of the cell. The size range from 0 to 250 μ was divided into twenty size classes at 12.5-μ intervals, and the percentage of oocytes in each size class was calculated.

Instantaneous relative growth rates were calculated for the post-pachytene oocytes according to Brody (1945, p. 508). The volume of the largest (and presumably first-produced) oocyte was calculated from the average frequency polygon for each collection; the volume was obtained from the diameter at the upper limit of the largest size class to contain at least 1% of the oocytes. On the assumption that the annual reproductive cycle follows essentially the same course year after year, we plotted the data point for 24 November 1973 as 24 November 1972 and the data point for 1 February 1974 as 1 February 1973. Volumes were plotted on a semilogaramithic scale against time in days after 15 November, the birth date of the first-produced oocyte, which was 8 μ in diameter at birth. Similarly, the volume of the smallest (and presumably last-produced) oocyte was calculated from the average frequency polygon for each collection; the volume was obtained from the diameter at the lower limit of the smallest size class to contain at least 1% of the oocytes. These volumes were plotted against time in days after 15 February 1973, the birth date of the last-produced oocyte, which was 8 μ in diameter at birth.

The total number of post-pachytene oocytes per ovary was calculated for females collected from March 1973 through October 1973. The total volume of all oocytes in the ovary (obtained by the method described three paragraphs above) was divided by the volume of an average oocyte; this latter quantity was obtained by calculating the volumes of the 50 oocytes used for the frequency polygon analysis and dividing their sum by 50. These calculated numbers of oocytes per ovary were compared with direct counts of the number of oocytes per ovary. Counts of all oocytes were made in a complete serial reconstruction of one ovary from a female collected on 1 March 1973. Also, counts of all oocytes were made by dissecting one ovary from each of five females collected on 2 October 1973. Both of these direct counting methods proved too time consuming for more extensive use.

The growth of the nucleus and nucleolus relative to oocyte size was determined from measurements of 100 post-pachytene oocytes that had been reconstructed serially. For each of the following collections, ten oocytes with the following ranges in diameter were measured: 14 December 1972 (8–40 μ), 1 March 1973 (30–80 μ), 7 May 1973 (45–100 μ), 1 July 1973 (80–150 μ), 2 September 1973 (120–170 μ), and 15 September 1973 (150–200 μ). For each of the following collections, twenty oocytes with the following ranges in diameter were measured: 24 September 1973 (158–213 μ, all with extracellular jelly and a smooth surface) and 2 October 1973 (157–216 μ, all with extracellular jelly and a dented surface).

The deposition of extracellular jelly around oocytes and the development of the dented oocyte surface were studied by plotting the frequency polygon for each female collected from 15 September through 2 October 1973. In these oocytes, it was noted whether annulate lamellae were present or absent; these organelles, which appear by light microscopy as basophilic inclusions in the oocyte cytoplasm, were actually identified as annulate lamellae by comparison of adjacent Epon thick sections with ultrathin sections examined by electron microscopy (Holland, unpublished).

Results

The first parts of this section deal with gross anatomy and tissues of the reproductive system; the spawning dates delimiting the annual reproductive cycle are also described. These topics, with their definitions, put the biometric results into context. The biometric results, in turn, provide a background for the cytological results.

Gross anatomy of C. japonica, *with special reference to the reproductive system*

The crinoid body consists of a central disc from which the arms radiate; each arm bears numerous side branches known as pinnules (Fig. 3 in Holland, 1975). For *C. japonica,* the 50 or so pinnules nearest the disc are called the genital pinnules, since each contains a gonad. Therefore, a large female with 40 arms would have about 2,000 ovaries, while a large male with 40 arms would have about 2,000 testes. All the gonads of a given animal are at virtually the same stage of development. Moreover, most of the gonads of a given animal are about the same size; only the most distally located gonads of each arm are markedly smaller than the other gonads (in the present study, these smaller gonads were never the ones sampled). Each gonad, which is elongate and approximately cylindrical, varies greatly in size during the annual reproductive cycle. When smallest, in November, the width is about 0.05 mm, and the length is about 6 mm. When largest, in early October, the width is about 1.2 mm, and the length is about 8 mm.

The tissues of the gonads

Both the ovary and the testis are composed of three layers arranged concentrically around a central, blind lumen. The *outer layer* of the gonad consists mainly of a sheet of visceral peritoneal cells. Muscle cells and nerve cells, which are also present, are very small and can be demonstrated clearly only by electron microscopy (Holland, 1971 and unpublished observations). For our purposes, the outer layer needs no further biometric or cytological description.

The *inner layer* of the gonad is the germinal epithelium, and its structure depends both on the sex of the animal and on the time of year. The inner layer of the ovary is made up of some combination of non-germinal cells, oogonia, spireme oocytes and post-pachytene oocytes. The cytodifferentiative sequence of these female germinal cells is: oogonia, spireme oocytes and post-pachytene oocytes. The inner layer of the testis is made up of some combination of non-germinal cells, spermatogonia, spermatocytes and spermatids. Spermatozoa are not a part of the inner layer, being located instead in the testicular lumen. The cytodifferentiative sequence of the male germinal cells is: spermatogonia, spermatocytes, spermatids and spermatozoa. In the ovaries and testes, several of the germinal cell populations undergo conspicuous fluctuations in volume, which will be described in the biometric part of the results section. All of the non-germinal and germinal cell types of the inner layer and lumen will be described in the cytological part of the results section.

Interposed between the outer and inner layers of the gonad is the *intermediate layer*, consisting of an inconspicuous matrix of haemal fluid in which are embedded the gonadal accessory cells. The size of the accessory cell population depends on the time of year, both in the ovary and in the testis. When enlarged, this cell population presses into the center of the gonad; at such times, the gonadal lumen, although reduced to a mere slit, always remains bounded by the inner layer. Fluctuations in the volume of the accessory cell population and the structure of the individual accessory cells will not be described in the present investigation.

Spawning dates delimiting the 1972–73 reproductive cycle

The population of *C. japonica* living in Koaziro Bay typically spawns each year on a single afternoon in October (Dan and Kubota, 1960). However, the annual reproductive cycle of 1972–73 was not typical, since it began with spawning on two consecutive afternoons (11 and 12 October 1972) and ended with spawning on at least five afternoons in October 1973. We did not anticipate such a complicated spawning pattern, and thus our sampling procedure left much to be desired. During the first half of October 1973, we collected crinoids every day or two. Some animals collected on 2, 3, 4, 17 and 18 October spawned after being brought into the laboratory on the day of collection. Moreover, some animals were observed spawning in the field during the afternoons of 3, 4 and 18 October; no field observations were made on 2 October, and those made on 17 October demonstrated no spawning animals. Random samples of animals collected on 7 and 11 October 1973 revealed that the crinoids spawning on 2, and 3 and 4 October accounted for about 40% of the males and about 40% of the females. After the spawnings of 17 and 18 October, a random sample collected on 22 October revealed that the remainder of the females had spawned; however, a few unspawned (or perhaps partially spawned) males were still present. By 8 November, all the collected males had spawned. Thus the 1972–73 reproductive cycle, which began in the second week of October 1972, ended for about 40% of the population in the first week of October 1973 and probably ended for the remaining 60% of the population in the third week of October 1973.

Volume fluctuations in the oocyte population and ovarian lumen

Figure 1A shows fluctuations in the volume of the population of post-pachytene oocytes and in the volume of the ovarian lumen. The population of oocytes increased in volume throughout the winter, spring and summer. This increase resulted from the enlargement of the individual oocytes, even though the total number of cells was decreasing from mid-winter through spawning in October (as will be demonstrated below). At spawning, the population volume decreased suddenly to a very small value, which declined to zero in the month that followed. The ovarian lumen, present but immeasurably small for much of the year, became enlarged in summer and was presumably filled with fluid (although some of the enlargement may have been a shrinkage artifact caused by fixation). In some ovaries, the enlarged lumen contained portions of degenerating oocytes, as illustrated on plate XV, Figure 4 in Dan and Dan (1941a). In the days immediately after spawning in October, the ovarian lumen remained somewhat distended; however, by a month after spawning, the lumen had become too small to measure.

Volume fluctuations in male germinal cell populations

Figure 1B shows fluctuations in the volumes of the germinal cell populations of the testis. For each cell type, the volume of the cell population is proportional to the number of cells present, since the sizes of individual cells do not vary greatly during the year. The population volume of the spermatogonia increased during winter, spring and early summer. An analysis of this volume increase by the method of Brody (1945, p. 513) revealed that, during this part of the year, the spermatogonia had a maximum mean cell cycle time of 35 days (we assumed that the growth fraction was unity and that no cell death occurred). Subsequently, during the latter part of August and during September, the population volume of the spermatogonia decreased rapidly and finally declined zero in the month after spawning. The population volume of the spermatocytes together with spermatids increased from zero in early July to maximum values during August and September. At spawning in early October, the volume fell suddenly and then declined to zero in the following weeks. The population volume of the spermatozoa was zero in early July, increased markedly during late summer, and then decreased suddenly at spawning. Finally, in the weeks that followed spawning, the volume declined to zero.

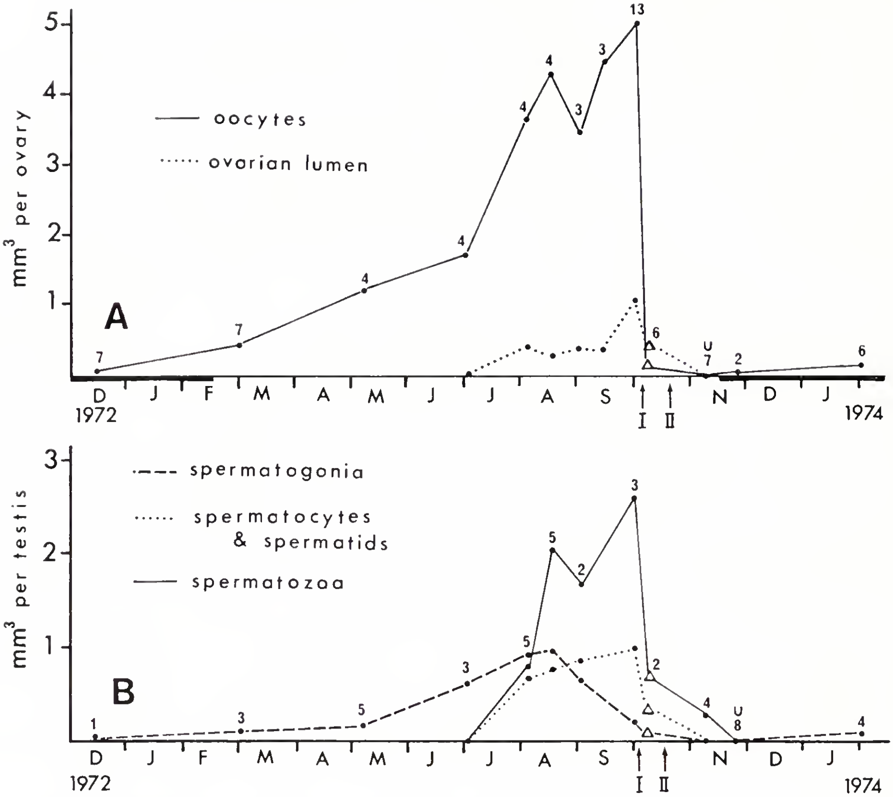


FIGURE 1. Volume fluctuations of gonadal regions during the annual reproductive cycle of *Comanthus japonica.* The number of individual animals averaged for each sample is shown above the date of collection. The spawnings of the first and third week of October 1973 are marked, respectively, by arrows I and II. For the triangular data points, only spawned animals in the collection were taken into account. For the data points labeled *U*, the animals were unsexable; (A) volume per ovary of the post-pachytene oocyte population and of the ovarian lumen. The thickened segments of the horizontal axis indicate the approximate interval during each annual cycle when precursors of the post-pachytene oocytes (namely, oogonia and spireme oocytes) were present in the ovary; (B) volume per testis of the male germinal cell populations.

Fluctuations in the size-frequency structure of the oocyte population

Changes in the size-frequency structure of the post-pachytene oocytes have been demonstrated by a frequency polygon analysis (Fig. 2). Each of the eleven average frequency polygons has been plotted against the date of collection. For most collections, the polygon of each individual female was approximately congruent with the average polygon; however, four of seven females collected on 1 March 1973 had distinctly bimodal frequency polygons. Otherwise, the individual polygons were unimodal, aside from relatively slight irregularities, which were presumably due to random sampling error.

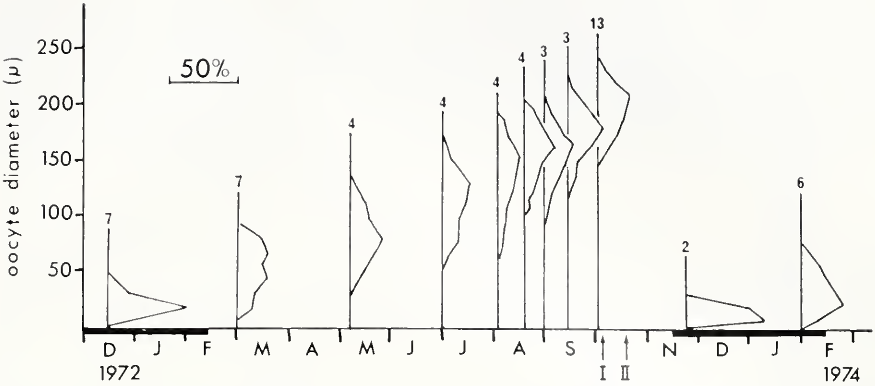


FIGURE 2. Fluctuation in the size-frequency structure of post-pachytene oocytes of *C. japonica.* Each of the eleven average frequency polygons was constructed from cytometric data from the number of females shown above the polygon. The thickened segments of the horizontal axis indicate the presence of oogonia and spireme oocytes. Arrows I and II mark, respectively, the spawnings of the first and third weeks of October 1973.

Within each ovary, the entire population of post-pachytene oocytes grew as a single generation (also called a cohort of oocytes). The growth of all oocytes as a single generation is rarely encountered in echinoderms, having previously been reported only for the asteroid *Patiriella regularis* (Crump, 1971) and possibly also for the asteroid *Asterias rubens* (Schlieper, 1957). This rare pattern of oocyte growth is certainly not a characteristic of crinoids in general, since the ovaries of most species contain more than one oocyte generation (Holland, unpublished observation).

In any female of *C. japonica* collected from late winter through early fall, the diameters of the largest and smallest oocytes differed by at least 80 μ. This wide range in oocyte diameters within each oocyte population was not an artifact caused by our sampling method, since similar ranges were found in random samples of unsectioned oocytes teased from fixed ovaries. Presumably, in each oocyte population, the largest cells were the first produced and thus the oldest, while the smallest cells were the last produced and thus the youngest; the following analysis of growth rates depends on the acceptance of the foregoing presumption.

Instantaneous relative growth rates of post-pachytene oocytes

The growth of two oocytes is shown in Figure 3. The curve at the left is for the largest (and presumably the first-produced) oocyte, and the curve at the right is for the smallest (and presumably the last-produced) oocyte. These cells, if spawned on 2 October, would be 321 and 229 days old respectively. The instantaneous relative growth rates (k) of the largest oocyte are as follows: 0.284 between 15 November and 24 November; 0.126 between 24 November and 14 December; 0.031 between 14 December and 1 March; and 0.012 between 1 March and 2 October. Similarly, instantaneous relative growth rates of the smallest

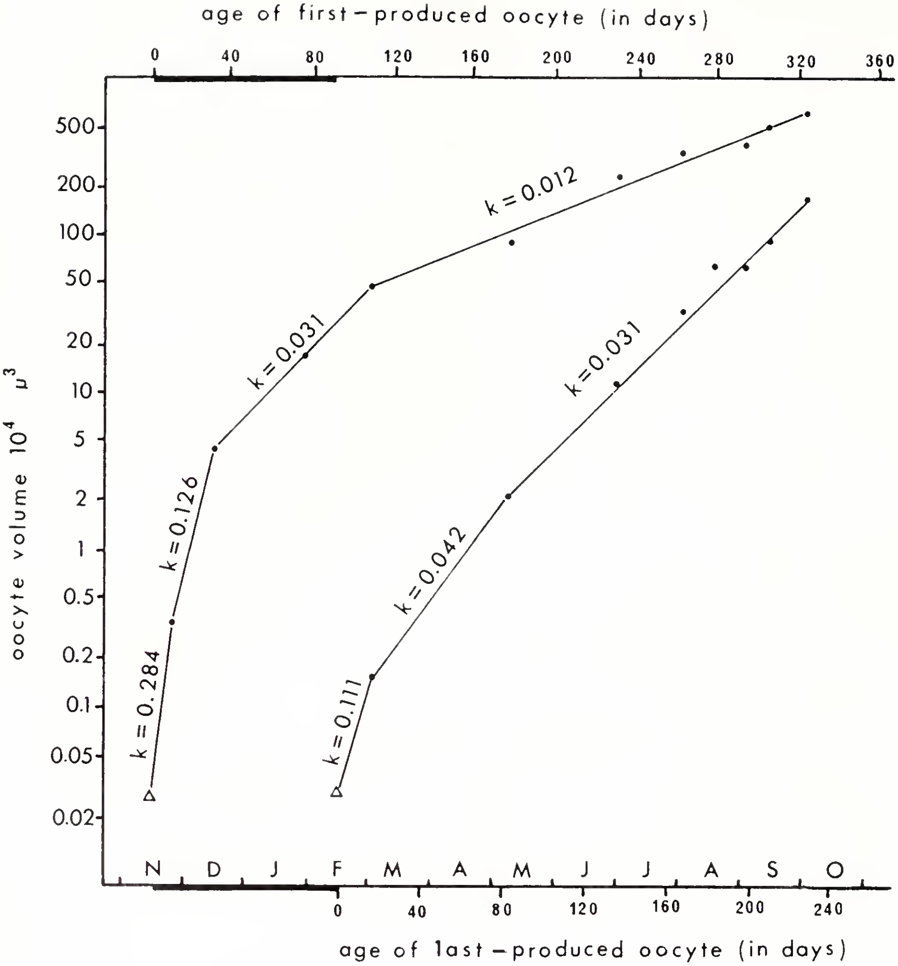


FIGURE 3. Instantaneous relative growth rates (k) of post-pachytene oocytes of *C. japonica*. The growth curve at the left is for the largest (and presumably the first-produced) oocyte, and the growth curve at the right is for the smallest (and presumably last-produced) oocyte. The triangular data point on each curve is the presumed volume on the birth date of the oocyte. The thickened segment of each horizontal axis indicates the presence of oogonia and spireme oocytes.

oocyte are as follows: 0.111 between 15 February and 1 March; 0.042 between 1 March and 7 May; and 0.031 between 7 May and 2 October.

The two curves of Figure 3 are not congruent. The last-produced oocyte, in comparison to the first-produced, has a slower early growth but a faster late growth. The reasons for these differences are not known. The tendency of the last-produced oocytes to catch up with the first-produced one during later growth is not great enough to prevent the wide range in oocyte diameters observed on the day of spawning. If one could plot growth curves for oocytes produced between mid-November and mid-February, one would no doubt obtain a family of curves falling between the two extremes plotted in Figure 3. All oocytes of *C. japonica*, no matter when they are produced, grow rapidly at first and then more slowly later. This pattern of oocyte growth contrasts markedly with that in *Strongylocentrotus purpuratus*, an echinoid in which the instantaneous growth rate is highest for medium-sized oocytes and not for small ones (Gonor, 1973b).

Changes in the total number of post-pachytene oocytes per ovary

The decline in the total number of post-pachytene oocytes per ovary is shown in Figure 4. Calculated numbers of oocytes per ovary were averaged (filled circles) and plotted against the date of collection. The eight collections represented were made from 1 March 1973 through 2 October 1973. In this seven-month period, during which no new cells were entering the cell population, the calculated number of oocytes per ovary declined from 3,360 to 1,348. By contrast, direct counts (triangles) revealed that, during the same period, the number of oocytes per ovary declined from 2,551 to 941. Evidently, the calculated values are somewhat too large, chiefly because the distal tapering of the ovary was not taken into account when the volume of the oocyte population was determined. In any case, it is safe to say that at least two to three times as many post-pachytene oocytes were originally produced as were present on the day of spawning. The decline in the number of oocytes was reflected by the presence of a few degenerating oocytes (and debris probably derived from them) in many of the ovaries sampled from March through October. It is not known if degeneration occurs at random in the oocyte population, irrespective of cell size. If one assumes that a large (40-armed)

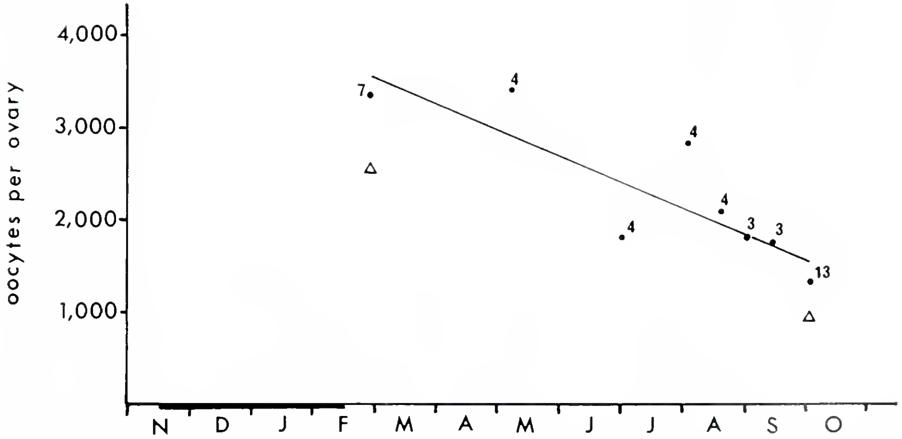
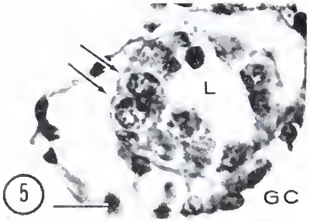
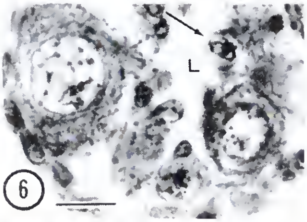
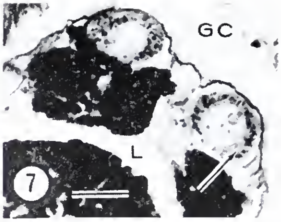
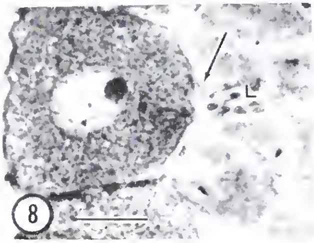


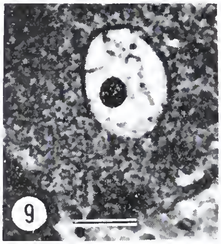
FIGURE 4. The decline in the total number of post-pachytene oocytes of *C. japonica* collected from early March through early October. The number of females averaged for each calculated number (filled circle) is shown above the date of collection; the regression line for these points was determined according to Sokal and Rholf (1969, chapter 14). The triangles show the number of oocytes determined by direct counts. The thickened segment of the horizontal axis indicates the presence of oogonia and spireme oocytes.

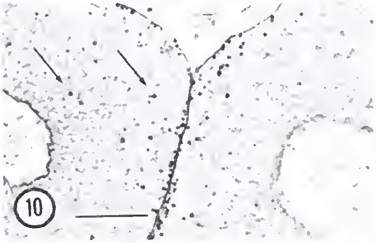


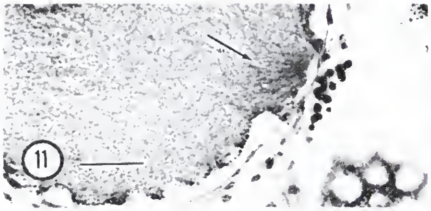


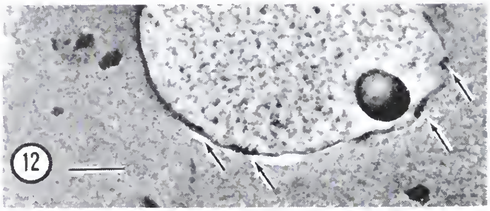


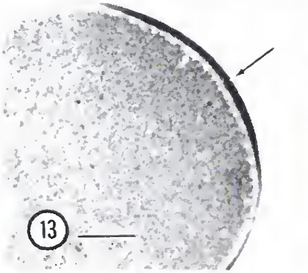


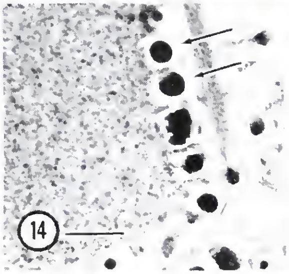


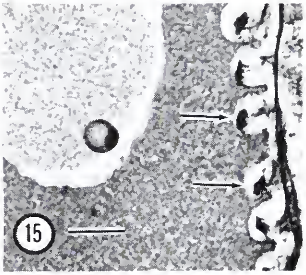


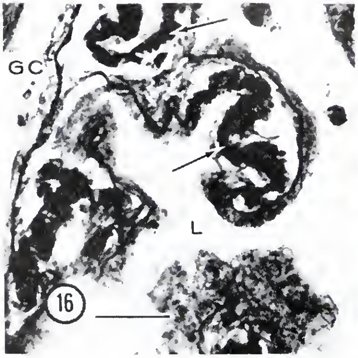












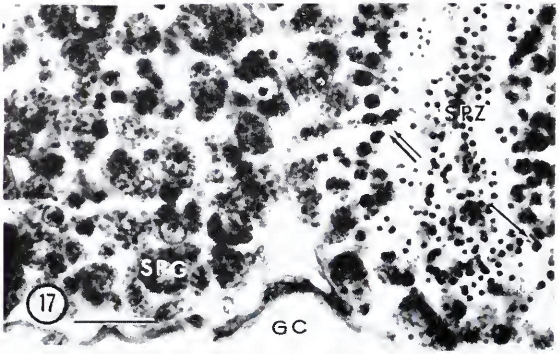


FIGURE 5. Cross section of a gonad of an unsexable animal collected 8 November 1973. Two gonial cells (arrows) are located in the inner layer of the gonad, which surrounds the

female has 2,000 ovaries which emit 1000 ova each, then a single female may spawn as many as two million ova.

The cytology of the cells of the inner layer of the unsexable gonad

The gonad, after spawning, passes through a brief phagocytic stage (which we will treat after the cells of the ripe gonads have been described) and enters a stage during which the sex of the animals cannot be established with certainty. During this unsexable stage, which lasts several weeks, a sex reversal by individual crinoids remains a possibility and should be investigated further. The inner layer of an unsexable gonad, like the one of 8 November (Fig. 5), consists of a simple epithelium of cuboidal cells. Some of these cells are about 8 μ across and contain

---

gonadal lumen (L). The intermediate layer of the gonad appears empty of cells, while the outer layer of the gonad is conspicuous. The gonad is surrounded by the genital coelom (GC). The scale line is 10 μ; haematoxylin and eosin.

FIGURE 6. Cross section of an ovary of a female collected 14 December 1972. A spireme oocyte (arrow) is located in the inner layer of the ovary, which surrounds the ovarian lumen (L). Several post-pachytene oocytes, like the one at the upper left, bulge away from the lumen. The genital coelom is labeled GC. The scale line is 10 μ; haematoxylin and eosin.

FIGURE 7. Cross section of an ovary of a female collected 14 December 1972. Yolk granules (arrow) are present in the cytoplasm of post-pachytene oocytes. The lumen and genital coelom are labeled L and GC, respectively. Darkly stained accessory cells are present in the intermediate layer of the ovary. The scale in line is 20 μ; PAS.

FIGURE 8. The periphery of an ovary from a female collected 1 March 1973. The arrow indicates the association zone between an oocyte and non-germinal cells of the inner layer of the ovary. Toward the right of the figure are several accessory cells and a portion of the narrow ovarian lumen (L). The scale line is 25 μ; haematoxylin and eosin.

FIGURE 9. The periphery of an ovary from a female collected 1 March 1973. Diplotene chromosomes are conspicuous in the nucleus of the oocyte. The scale line is 20 μ; haemotoxylin and eosin.

FIGURE 10. Oocytes of a female collected 4 August 1973. Cortical granules (arrows) are scattered in the cytoplasm and are also abundant just beneath the plasma membrane. The scale line is 30 μ; azure A.

FIGURE 11. Dented oocytes of a female collected 24 September 1973. Cytoplasmic fibers (arrow) radiate from the region where a dented oocyte is associated with non-germinal cells of the inner layer of the ovary. A dented oocyte in tangential section is at the bottom right. The scale line is 25 μ; azure A.

FIGURE 12. A dented oocyte of a female collected 2 October 1973. Diakinesis chromosomes (arrows) lie at the periphery of the nucleus. The dark patches in the cytoplasm are annulate lamellae. The scale line is 25 μ; haematoxylin and eosin.

FIGURE 13. An oocyte of a female collected 24 September 1973. The extracellular jelly is in the form of a continuous layer (arrow). The scale line is 25 μ; PAS.

FIGURE 14. Dented oocytes of a female collected 27 September 1973. The extracellular jelly is mostly in the form of jelly spheres (arrows). The scale line is 25 μ; alcian blue and PAS.

FIGURE 15. A dented oocyte of a female collected 2 October 1973. The extracellular jelly is in the form of jelly clumps (arrows). The scale line is 25 μ; alcian blue.

FIGURE 16. The periphery of an ovary from a spawned female collected 7 October 1973. The ovarian lumen is labeled L, and the genital coelom is labeled GC. The chorions (arrows), which formerly surrounded the oocytes, have been left behind at ovulation in the inner layer of the ovary. The scale line is 30 μ; alcian blue and PAS.

FIGURE 17. The periphery of a testis from a male collected on 4 August 1973. Most of the cells at the left are spermatogonia (SPG). At the right are several spermatocytes (twin arrow), a spermatid (single arrow) and numerous spermatozoa (SPZ). The genital coelom is labeled GC. The scale line is 15 μ; haematoxylin and PAS.

a spherical nucleus about 6 μ in diameter. The nucleus contains one or two nucleoli about 1.5 μ in diameter and is surrounded by a thin shell of clear cytoplasm. It is reasonable to assume that these nucleolate cells are gonial cells. In addition to the gonial cells, the inner layer includes non-germinal cells characterized by a small amount of cytoplasm surrounding an oval nucleus (about 5 μ × 3 μ) that lacks a conspicuous nucleolus.

The cytology of the inner layer of the ovary until late September

By late November, ovaries are unequivocally recognizable, since their inner layers by then contain definitive female germinal cells. From late November through early February, the inner layer is made up of non-germinal cells, oogonia, spireme oocytes and post-pachytene oocytes. The oogonia, which resemble the gonial cells described in the preceding paragraph, differentiate into spireme oocytes (a term which covers the leptotene through pachytene stages of the first meiotic prophase). Each spireme oocyte is about 7 μ in diameter and has an anucleolate nucleus containing a tangle of threadlike chromatin (Fig. 6, arrow). Spireme oocytes give rise to post-pachytene oocytes (a term which covers the diplotene, diffuse, and diakinesis stages of the first meiotic prophase). Post-pachytene oocytes are about 8 μ in diameter when newly produced, but most are much larger. In the nucleus, one conspicuous nucleolus is invariably present. As post-pachytene oocytes enlarge, they soon bulge outward, away from the ovarian lumen (Figs. 6 and 7). From late February through spawning in October, the inner layer of the ovary consists only of non-germinal cells and post-pachytene oocytes.

Although the post-pachytene oocytes bulge away from the ovarian lumen, the inner edge of each oocyte remains in intimate association with the non-germinal cells of the inner layer (Figs. 8 and 11). Thus oocytes always belong to the inner layer of the ovary in *C. japonica*. In other crinoid species, oocytes have often been considered part of the intermediate layer of the ovary; this may well be an error of omission caused by the failure of previous workers (including Holland, 1971) to recognize the inconspicuous zone of cell-to-cell association. This zone was seen by Cuénot (1891) and by Dan and Dan (1941a), who referred to it as the oocyte stalk; however, those authors did not demonstrate that the base of the "stalk" was associated with non-germinal cells of the *inner* layer of the ovary. There is no light microscopic evidence that the association zone is the site of a massive transfer of nutrients from non-germinal cells to the oocyte.

From the birth date of a post-pachytene oocyte (which may be any time between 15 November and 15 February) until late September, cell structure depends on cell size. In Figure 18A (filled circles), the nuclear diameter has been plotted against the diameter of oocytes sampled prior to late September. The curve, by inspection, has an inflection at an oocyte diameter of 75 μ. The points to the left and right of the inflection are respectively fitted by the following regression lines: $y = 0.343x + 5.07$ and $y = 0.222x + 14.75$. Post-pachytene oocytes less than about 100 μ in diameter are in the diplotene stage, and their nucleoplasm contains strands of basophilic material (Figs. 6 and 9) that are presumably chromosomes. Oocytes larger than about 100 μ in diameter are in the diffuse stage (usage of Rhoades, 1961), and the nucleoplasm consists only of diffuse, flocculent material (Fig. 10).

The nucleolus of each oocyte is eccentrically located in the nucleus. In Figure 18B (filled circles) the nucleolar diameters have been plotted against the diameters of oocytes sampled prior to late September. The curve, by inspection, has an inflection at an oocyte diameter of 50 μ. The points to the left and right of the inflection are respectively fitted by the following regression lines: $y = 0.117x + 0.84$ and $y = 0.038x + 4.09$. Until late September, the nucleolus always consists of a spherical core surrounded concentrically by a thin cortex. Both core and cortex are strongly basophilic, the latter slightly more so than the former. Nucleolar staining with mercuric bromphenol blue varies in intensity from oocyte to oocyte; however, in general, the core stains more intensely than the cortex. In oocytes greater than about 75 μ in diameter, alcian blue stains the nucleolar core but not the cortex; thus, surprisingly, the core apparently contains nonsulfated acid mucopolysaccharide. No part of the nucleolus is ever stained by PAS.

Eosinophilic yolk granules begin to appear when post-pachytene oocytes reach a diameter of about 12 μ; soon thereafter, the yolk granules become ubiquitously and abundantly distributed throughout the oocyte cytoplasm. Each granule varies from about 0.5 μ to 2.5 μ in diameter. Cytochemically, the yolk granules stain intensely with PAS (Fig. 7) and moderately with mercuric bromphenol blue; these reactions indicate an abundance of neutral mucopolysaccharide and protein respectively. Since alcian blue and azure A do not stain the granules, little or no acid mucopolysaccharide is present. Vitellogenesis in *C. japonica* is very precocious in comparison to that in most other animals. Such precocious vitellogenesis is not characteristic of other crinoids that have been studied; in these, the first part of oocyte growth includes a long period of basophilic cytoplasm in which yolk granules are rare or absent (Chubb, 1906; Vannini, 1953; Urbani, 1955; Davenport and Davenport, 1966; Holland, 1971).

Cortical granules begin to appear in the cytoplasm of oocytes larger than 115 μ in diameter, excepting a few such cells in the sample of 7 May 1973. The cortical granules, which range in diameter from 1 μ to 3 μ, are stained dark blue (orthochromasia) with azure A (Fig. 10). Mercuric bromphenol blue stains the cortical granules only slightly darker than the yolk granules. Neither alcian blue nor PAS stain the cortical granules at all. In the collections of July, August and the first part of September, some cortical granules were scattered widely throughout the oocyte cytoplasm (Fig. 10, arrows), while the rest of the cortical granules were located just beneath the plasma membrane. There is little doubt that the cortical granules scattered in the cytoplasm are being produced there; then, after having been produced, they migrate to the extreme periphery of the oocyte where they accumulate.

The inner edge of each post-pachytene oocyte, as already described, remains in intimate association with non-germinal cells of the inner layer of the ovary. The oocyte cytoplasm, just subjacent to this association zone, often contains fibers which radiate toward the center of the cell. These fibers (some of which are visible, but not conspicuous in Fig. 8) stain darkly with haematoxylin and also with azure A (showing orthochromasia). Although the function of these fibers is not known, they are evidence of an early cytoplasmic polarity in the post-pachytene oocytes.

The oocyte surface appears smooth by light microscopy in collections made before late September. Around oocytes that have reached about 75 μ in diameter, an extracellular coat, which we will call the chorion, becomes faintly stainable with alcian blue. This chorion becomes more conspicuous around larger oocytes, but never exceeds half a micron in thickness; it continues to stain with alcian blue and also stains weakly with PAS, mercuric bromphenol blue and azure A (showing beta metachromasia). These cytochemical reactions indicate that the chorion includes some protein and acid mucopolysaccharide. Even when well-developed, the chorion never intervenes between the oocyte and the adjacent non-germinal cells of the inner layer; instead, each chorion is probably continuous with the basal lamina underlying the non-germinal cells of the inner layer (this relationship will be discussed further below).

The cytology of the inner layer of the ovary from late September to spawning

Between late September and spawning in October, the inner layer of the ovary still consists only of non-germinal cells and post-pachytene oocytes. By this time, the nuclei of the several dozen non-germinal cells adjacent to each oocyte become more spherical and more basophilic than before (Fig. 11). Oocyte structure no longer depends on cell size; instead, all the oocytes in a given ovary begin to differentiate almost simultaneously, even though the diameters of the largest and smallest cells in the population differ by about 80 μ. During the final weeks of oogenesis, conspicuous morphological changes occur both in the nucleus and in the cytoplasm.

A disproportional swelling of the oocyte nucleus begins after mid September (Fig. 18A). The swelling is significant, since the confidence limits of the following regression lines do not overlap: $y = 0.222x + 14.75$ (15 September); $y = 0.212x + 30.65$ (24 September); and $y = 0.128x + 63.19$ (2 October). Moreover, in the collections from 27 September through 2 October, chromosomes reappear in most of the oocytes (Fig. 12, arrows). Such oocytes have entered the diakinesis stage of the first meiotic prophase. The diakinesis chromosomes are short, basophilic structures located at the periphery of the nucleus; one of them is typically associated with the nucleolus. Elsewhere in the nucleus, the nucleoplasm consists of flocculent material.

The oocyte nucleolus also begins disproportional swelling after mid September (Fig. 18B). The swelling is significant, since the confidence limits of the following regression lines do not overlap: $y = 0.038x + 4.09$ (15 September); $y = 0.002x + 11.85$ (24 September); and $y = 0.018x + 13.31$ (2 October). By 2 October, the shape of each nucleolus has become slightly ovoid (Fig. 12), but not as conspicouslyso as some of the nucleoli in the figures of Dan and Dan (1941a). Structurally, the nucleolus no longer consists of a core surrounded concentrically by a thin cortex. Instead, the former core, identifiable by its affinity for alcian blue (Fig. 15), is now located at one side of the nucleolus; the core material remains basophilic and still stains with mercuric bromphenol blue. The remainder of the nucleolus probably corresponds to the former cortex; however, this presumed cortical material, in comparison to the former cortex, is much less basophilic, but stains more intensely with mercuric bromphenol blue. Some, but not all, of the nucleoli at this stage contain one or several nucleolar vacuoles (Fig. 15).

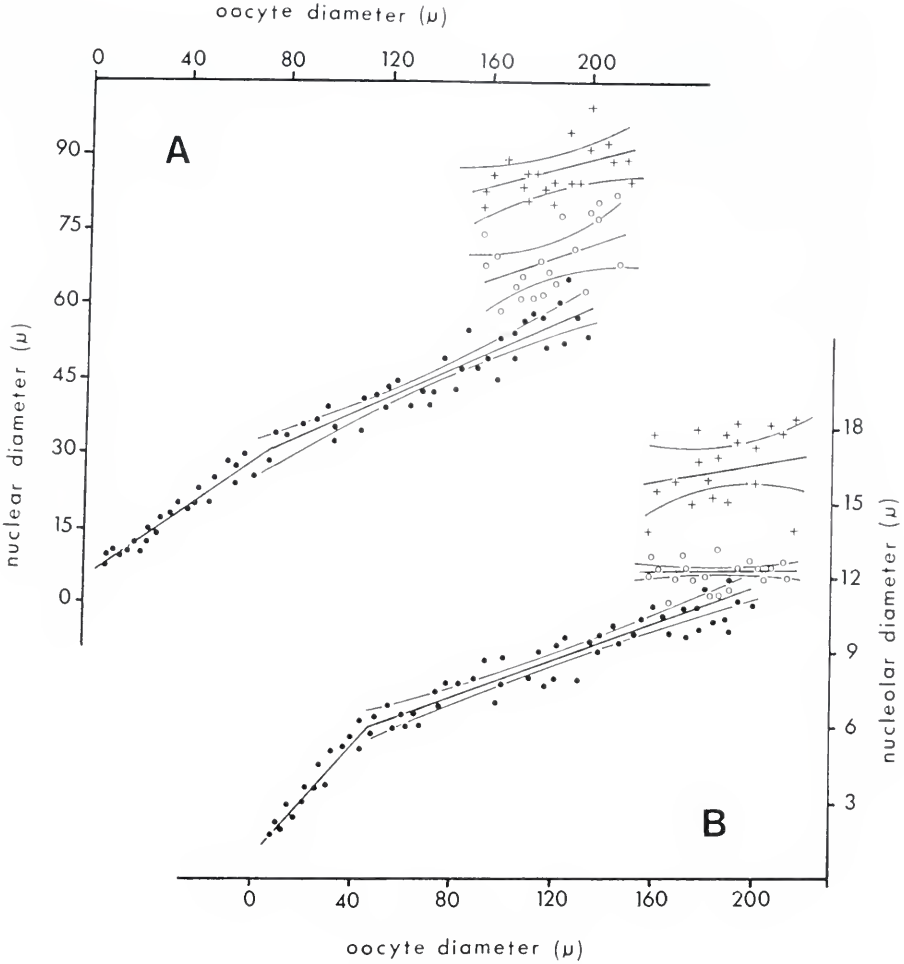
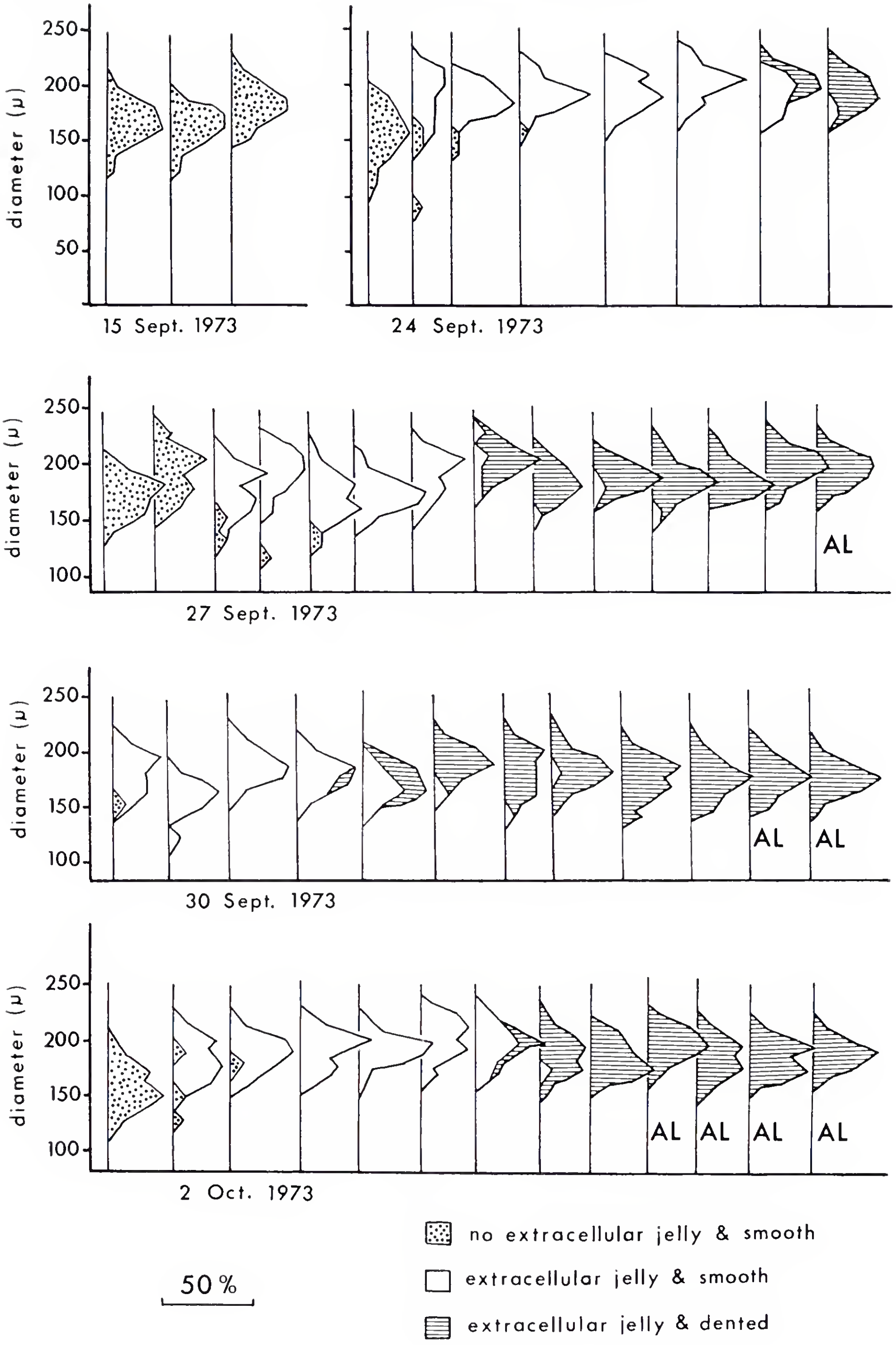


FIGURE 18. (A) The growth of the nucleus relative to the size of the post-pachytene oocyte; (B) the growth of the nucleolus relative to the size of the post-pachytene oocyte. In both graphs, the filled circles are diameters measured from specimens collected from 14 December 1972 through 15 September 1973; the open circles are diameters measured from specimens collected on 24 September 1973; and the crosses are diameters measured from specimens collected on 2 October 1973. The regression lines and 95% confidence limits have been calculated according to Sokal and Rholf (1965, chapter 14).

The cortical granules, from 15 September through 2 October, are located just beneath the plasma membrane of the oocyte (Fig. 11). Their absence from deeper regions of the cytoplasm presumably reflects the cessation of cortical granule production by mid September.

Annulate lamellae were present in the oocyte cytoplasm of one female collected on 27 September. By light microscopy, annulate lamellae (which were identified as such by our unpublished electron microscopy) appear in the cytoplasm as baso-


diameter (μ)
250
200
150
100
50
15 Sept. 1973
24 Sept. 1973
27 Sept. 1973
AL
30 Sept. 1973
AL
AL
2 Oct. 1973
AL
AL
AL
AL
50 %
no extracellular jelly & smooth
extracellular jelly & smooth
extracellular jelly & dented

philic patches ranging from about 3 μ to 15 μ in average diameter (Fig. 12). In oocytes of one female of 27 September, some annulate lamellae were closely associated with the edge of the nuclear envelope, possibly being elaborated there. In the collections of 30 September and 2 October, annulate lamellae were present in the oocytes of two and four females respectively (Fig. 19).

Extracellular jelly appeared around the oocytes of most females in the population during the third week of September 1973 (Fig. 19), apparently regardless of whether the females were destined to spawn in the first or the third week of October. Within a given ovary, all the oocytes became surrounded by jelly almost simultaneously in the context of the whole course of oogenesis; actually, the smallest oocytes tended to become surrounded by jelly a few hours or days after the larger oocytes.

The extracellular jelly, when it first appeared, was a layer about 7 μ thick between the plasma membrane and the chorion. This layer (Fig. 13, arrow) was an unbroken sheet of homogeneous material, often limited to the oocyte hemisphere nearest the ovarian lumen. This jelly stained intensely with PAS, but remained unstained by azure A and alcian blue; thus neutral mucopolysaccharide was present, but acid mucopolysaccharide was undetectable at this stage. A strong reaction with mercuric bromphenol blue indicated that protein was also present. The origin of the extracellular jelly is not known with certainty; however, it seems likely that most or all of the jelly is produced intracellularly and secreted by the oocyte itself.

The formerly smooth oocyte surface became dented (Fig. 19) a few days after the advent of the extracellular jelly, at least in those females destined to spawn in the first week of October. Within a given ovary, all the oocytes became dented within a relatively short time of one another (perhaps within hours or, at most, a few days). Denting began with the appearance of hundreds of pits in the oocyte surface (Fig. 11). The diameter of each pit was roughly 15 μ to 20 μ and the depth was about 10 μ (Figs. 14 and 15). Such pits were never widely scattered over the oocyte surface; instead they were almost contiguous, as can be seen from the tangentially sectioned oocyte at the bottom right in Fig. 11. In some oocytes of 24 September, the pits were relatively shallow and had presumably just been formed. Occasionally, an oocyte was seen with one smooth hemisphere and one shallowly pitted hemisphere; this pattern indicates that denting might start at one point on the surface and spread from there. The cytoplasmic fibers (Fig. 11, arrow), which are present as denting begins, vanish shortly thereafter; it is not known if they play a role in surface denting.

For a given oocyte, the advent of the dented surface was closely correlated with the breaking up of the extracellular jelly into numerous isolated spheres, each about 10 μ in diameter (Fig. 14). The histochemistry of most of these spheres resembled that of the jelly layer of smooth oocytes; neutral mucopolysaccharide and protein were conspicuous components, but no acid mucopolysaccharide was detectable. It is not known with certainty whether the jelly spheres caused denting or the denting caused jelly spheres. To picture the first possibility,

---

FIGURE 19. The size-frequency structure of the post-pachytene oocytes of each female collected from 15 September 1973 through 2 October 1973; frequency polygons of females having annulate lamellae in their oocytes have been labeled AL.

one could imagine that the jelly layer surrounding a smooth oocyte spontaneously broke into numerous jelly spheres, and that the oocyte cytoplasm then intruded into the spaces between them. To picture the second possibility, one could imagine that the jelly layer surrounding a smooth oocyte was divided into numerous jelly spheres by cytoplasmic ridges actively elevating from the oocyte surface. The second possibility, that denting causes jelly spheres, is the more likely, since a dented area of oocyte surface may occasionally be overlain by an unbroken jelly layer. One additional mode of jelly sphere formation might be the secretion of jelly into a pre-existing pit after denting has been completed.

The jelly spheres, soon after being formed, undergo further morphological and histochemical transformation. Each sphere, which consists of relatively homogeneous material (Fig. 14, arrows) appears to be converted into a condensed clump of relatively fibrous material (Fig. 15, arrows). These fibrous jelly clumps stain intensely with alcian blue, but do not stain with PAS or azure A; such histochemical properties define a nonsulfated acid mucopolysaccharide (Spicer, 1963). Mercuric bromphenol blue fails to stain the jelly clumps, indicating that protein is absent or masked from reacting with the stain. A given pit may sometimes contain both clump and sphere material; in such cases, the clump material is always located nearest the bottom of the pit. Each jelly clump is probably produced by an extracellular conversion of the jelly sphere's neutral mucopolysaccharide to nonsulfated acid mucopolysaccharide, perhaps under the influence of enzymes released from the oocyte.

From Figure 19, it is reasonable to assume that most of the females which failed to spawn on 2, 3 and 4 October had smooth oocytes with extracellular jelly. Such oocytes were still present in the eight unspawned females collected on 7 October. Of seven unspawned females collected on 11 October, four had smooth oocytes with extracellular jelly, and three had dented oocytes with extracellular jelly. Thus the oocytes of the unspawned females were apparently becoming dented about a week before the spawnings of 17 and 18 October. It is probable, therefore, that oocytes become dented a week before spawning, regardless of whether spawning took place in the first or in the third week of October. Other oocyte development dependent on the time before spawning might be the production of annulate lamellae and the appearance of diakinesis chromosomes (unfortunately, these phenomena were not studied just prior to the spawnings of the third week of October).

In the hours leading up to spawning, ovulation occurs, and the oocytes mature into ova; these phenomena will only be summarized here, since they are more appropriately demonstrated by electron microscopy (Holland, unpublished observations) than by light microscopy. At ovulation, the oocytes squeeze between the non-germinal cells of the inner layer of the ovary and enter the lumen. In the ovarian lumen, the oocytes quickly pass through the two meiotic divisions and become ova. Soon thereafter, the ova are expelled from the lumen through a simultaneous rupture of the gonadal wall and the lateral body wall of the pinnule.

The cytology of the inner layer of the ovary soon after spawning; the phagocytic stage

At ovulation, as the oocytes pass from the inner layer to the lumen, the chorions of the oocytes are left behind, much collapsed and thickened, as a part of the inner layer of the ovary (Fig. 16, arrows). The collapsed chorions are histochemically the same as the ones that surrounded the unovulated oocytes. In the days after spawning, one can see that the collapsed chorions are continuous with a basal lamina underlying the non-germinal cells of the inner layer of the ovary. Presumably, this basal lamina is always present, but is too thin during much of the year to be demonstrable by light microscopy. Collapsed chorions can be recognized in the ovaries for several weeks after spawning, but then disappear, probably by destruction *in situ* by non-germinal cells of the inner layer or intermediate layer.

Most spawned ovaries retain about a dozen oocytes and ova; the unspawned oocytes remain a part of the inner layer, and the unspawned ova remain free in the ovarian lumen. Within a few weeks after spawning, all unspawned germinal cells have broken down, and their remains have been taken up phagocytically by the non-germinal cells of the inner layer. During this phagocytic stage, these non-germinal cells range in shape from cuboidal to low columnar.

The cytology of the inner layer of the testis until spawning

The testes, after spawning, pass through a phagocytic stage (described below) and an unsexable stage (which has already been described) . By mid December, the unsexable stage ends, and the testes become unequivocally recognizable, since definitive spermatogonia are present along with non-germinal cells in the inner layer. Definitive speratogonia, while generally resembling gonial cells of unsexable animals, are characterized by the presence of small cytoplasmic granules. Such granules, which range in diameter from about 0.5 μ to 1.5 μ, stain moderately with PAS, weakly with mercuric bromphenol blue and not at all with azure A or alcian blue. Comparable granules have never been reported in the spermatogonia of other echinoderms.

Throughout the winter, spring and early summer, the spermatogonia divide mitotically without differentiating into more advanced germinal cells. During this part of the annual reproductive cycle, as already mentioned, the maximum mean cell cycle time of the spermatogonia is 35 days. As a result of this cell division, the inner layer of the testis becomes about eight spermatogonia thick by 1 July. A few of the spermatogonia are about twice the size of the others; these larger cells might be about to enter mitosis. The testis of *C. japonica* might contain both multiplying spermatogonia and terminal spermatogonia (in the terminology of Giese and Pearse, 1974) ; however, these two cell varieties are indistinguishable by morphological criteria. Some non-germinal cells probably continue to be present in the inner layer, but we could not find them among the abundant spermatogonia.

By the collection of 4 August, the spermatogonia have begun to differentiate into more advanced germinal cell types (Fig. 17). The inner layer of the testis now includes spermatogonia, spermatocytes (presumably both primary and secondary), and spermatids, while the testicular lumen contains some spermatozoa. Thus, the cytodifferentiative sequence from spermatogonium to spermatozoa is completed in a month at the most. In the testis of *C. japonica*, the spermatocytes occupy the more luminal parts of the inner layer and appear as densely basophilic nuclei about 4 μ in diameter surrounded by clear cytoplasm (Fig. 17, twin arrow). The spermatocytes are often seen in meiotic divisions. The spermatids, which occur right next to the lumen, appear as dense nuclei about 2.5 μ in diameter

(Fig. 17, single arrow). In the testicular lumen, the heads of the spermatozoa are dense, basophilic spheres just under 2 μ in diameter.

Spermatogonial differentiation continues throughout August, September and early October; thus, at spawning, the first-produced spermatozoon is roughly ten weeks older than the last-produced spermatozoon. Shortly before spawning in October, the inner layer of the testis consists mainly of spermatocytes and spermatids, and spermatogonia are much less abundant than earlier in the summer. Apparently, the rate of spermatogonial differentiation has become far greater than the rate of spermatogonial division, and the cell population has, therefore, been depleted.

The cytology of the inner layer of the testis soon after spawning; the phagocytic stage

When males spawn, the cells expelled from the testis apparently include not only spermatozoa, but also many of the less advanced germinal cells of the inner layer (Fig. 1B). After spawning, the testis invariably retains some spermatozoa in the lumen. During the next few weeks, non-germinal cells become conspicuous in the inner layer; they appear to engulf spermatozoa from the lumen and probably also engulf the less advanced germinal cells remaining in the inner layer. After a few weeks of phagocytosis, no germinal cells of any kind, not even spermatogonia, can be found in the testis.

DISCUSSION

In November, gonial cells first appear in the gonads of *Comanthus japonica.* This event, which establishes a rough gametogenic synchrony among the individual animals of the population, is probably initiated in both sexes by an environmental signal dependent on the time of year (perhaps shortening photoperiod or falling sea temperature). Unfortunately, it is not presently known whether the advent of the gonial cells is a matter of cell migration or cell differentiation. The gonial cells might migrate into the gonad from elsewhere in the animal (as proposed by Hamann, 1888, p. 83), or they might differentiate *in situ* from non-germinal cells of the inner layer of the gonad. In the testes, spermatogonia divide mitotically throughout the winter, spring and early summer without entering meiosis (*i.e.*, differentiating into spermatocytes). The deveolpmental block between spermatogonia and spermatocytes is not overcome until July, August and September. In the ovaries, by contrast, the oogonia undergo relatively little mitotic division and soon enter meiosis (*i.e.*, differentiate into oocytes). This differentiation continues through the winter until mid February, by which time the supply of oogonia apparently becomes exhausted. Because male and female germ cells do not follow parallel courses after the gonial cells have appeared, it is reasonable to assume that one set of controls operates in males and another operates in females during much of the year.

Oocyte cytology depends on cell size throughout the winter, spring and summer; moreover, there is neither a suspension of oocyte growth nor a sudden increase in the instaneous relative growth rate. These characteristics indicate that much of oocyte growth is autodifferentiative (in the sense of Charniaux-Cotton, 1973). Then, from late September until spawning in October, oocyte cytology no longer depends on cell size; instead, all the oocytes in a given ovary begin to differentiate almost simultaneously, in spite of the 80 μ range in their diameters. Such a response pattern is a good indication that environmental signals are acting on the entire cell population via nervous and endocrine mechanisms. Unfortunately, nothing definite is presently known about reproductive endocrinology in crinoids. A study of the reproductive endocrinology of *C. japonica* would be of special interest because of the high degree of spawning synchrony in this species.

On the day of spawning each year, a large female specimen of *C. japonica* emits a total of about two million ova. In making this calculation, we ignored the few unspawned ovaries that can usually be found scattered at random among the spawned ovaries after the ova have been emitted (Dan and Dan, 1941b); we also ignored the dozen or so gametes remaining in each spawned ovary. In spite of these sources of error, the fecundity of a female can be determined for *C. japonica* far more accurately than for most other animals broadcasting large numbers of eggs into the sea water. Under good laboratory conditions, nearly all the spawned ova are fertilizable, in spite of the wide range in cell diameters. It is not presently known if the size of a zygote at the start of development ultimately influences the individual's chance of survival during the subsequent life history.

Within a few minutes after fertilization, the zygote of *C. japonica* becomes surrounded by an elaborate fertilization membrane, which has been described by Holland and Jespersen (1973). The pattern of ridges on the outer surface of the fertilization membrane is very reminiscent of the surface topography of the dented oocyte. There can be little doubt that the oocyte surface is a template that somehow imposes its form on the fertilization membrane. The details of how the surface pattern is transferred during fertilization and the cortical reaction have yet to be elucidated.

The present investigation was made possible by the cordial hospitality of Director Hideshi Kobayashi and the staff of the Misaki Marine Biological Station of the University of Tokyo. Special thanks are due to Mrs. Linda Holland for doing our statistics and to Dr. Shonan Amemiya, Dr. Bo Fernholm and Dr. Tomoyuki Ichikawa for helping us to collect and make field observations. Our paper was greatly improved by the critical readings of Professor R. B. Clark, Dr. P. J. W. Olive and Dr. J. S. Pearse.

SUMMARY

1. Periodic sampling of a Japanese population of an unstalked crinoid, *Comanthus japonica*, demonstrated an annual reproductive cycle delimited by spawning in October.

2. In both sexes, the first weeks after spawning were a time of phagocytosis of unspawned germinal cells by non-germinal cells of the inner layer of the gonad.

3. In November, gonial cells made their appearance in the inner layer of the gonad, which was unsexable for several weeks.

4. In females, during the latter part of November, oogonia began differentiating into oocytes and continued to do so until mid February; this resulted in an

oocyte population in which the largest cells were three months older than the smallest cells.

5. During the winter, spring and summer, the oocyte population grew as a single generation until spawning in October; although there was some tendency for the smaller oocytes to catch up with the larger ones, oocyte diameters ranged from about 145 μ to 225 μ on the day of spawning. The instantaneous relative growth rates of all oocytes were high at first and then decreased during later growth.

6. In each ovary, at least two to three times as many oocytes were initially produced as were finally present just before spawning; a large female emitted about two million gametes on the day of spawning each year.

7. Prior to late September, oocyte morphology depended on oocyte size. The following changes occurred at the following oocyte diameters; yolk granule synthesis started at 12 μ; the diplotene chromosomes disappeared at 100 μ; and cortical granule synthesis started at 115 μ.

8. From late September to spawning in October, all the oocytes in an ovary began to differentiate almost simultaneously, irrespective of their size. During this short period, the nucleus and nucleolus swelled conspicuously, extracellular jelly appeared, and the oocyte surface became dented with hundreds of pits, each about 10 μ deep. Finally, several days before spawning, annulate lamellae appeared in the cytoplasm, and diakinesis chromosomes appeared in the nucleus.

9. In males, the spermatogonia divided mitotically throughout the winter, spring and early summer without differentiating into more advanced germinal cell types; during this period, the maximum mean cell cycle time was about 35 days. Subsequently, from mid summer through spawning in October, the spermatogonia differentiated, via spermatocytes and spermatids, into spermatozoa.

LITERATURE CITED

Brody, S., 1945. *Bioenergetics and Growth.* Reinholdt Publishing Co., New York, 1023 pp.

Charniaux-Cotton, H., 1973. Introduction à l'ovogenèse chez les invertébrés. *Annales de Biologie Animale, Biochimie, Biophysique,* **13**: 13–19.

Chia, F. S., 1968. Some observations on the development and cyclic changes of the oöcytes in a brooding starfish, *Leptasterias hexactis.* *J. Zool.,* **154**: 453–461.

Chubb, G. C., 1906. The growth of the oocyte in *Antedon:* a morphological study in the cell-metabolism. *Phil. Trans. Royal Society London Series,* **B198**: 447–505.

Crump, R. G., 1971. Annual reproductive cycles in three geographically separated populations of *Patiriella regularis* (Verrill), a common New Zealand asteroid. *J. Exp. Mar. Biol. Ecol.,* **7**: 137–162.

Cuénot, L., 1891 Études morphologiques sur les échinodermes. *Arch. Biol.,* **11**: 313–680.

Dan, J. C., and K. Dan, 1941a. Early development of *Comanthus japonicus.* *Jap. J. Zool.,* **9**: 565–574.

Dan, K., and J. C. Dan, 1941b. Spawning habit of the crinoid, *Comanthus japonicus.* *Jap. J. Zool.,* **9**: 555–564.

Dan, K., and H. Kubota, 1960. Data on the spawning of *Comanthus japonica* between 1937 and 1955. *Embryologia,* **5**: 21–37.

Davenport, R., and J. C. Davenport, 1966. A cytochemical study of cytoplasmic basic proteins in echinoderm oogenesis. *Exp. Cell Res.,* **42**: 429–437.

Fenaux, L., 1968. Maturation des gonades et cycle saisonnier des larves chez *A. lixula, P. lividus,* et *P. microtuberculatus* (Echinides) à Villefranche-sur-Mer. *Vie et Milieu (Ser. A),* **19**: 1–52.

Fenaux, L., 1970. Maturation of the gonads and seasonal cycle of the planktonic larvae of the ophiuroid *Amphiura chiajei* Forbes. *Biol. Bull.*, **138**: 262–271.

Fenaux, L. 1972. Evolution saisonnière des gonades chez l'ophiure *Ophioderma longicauda* (Retzius), Ophiuroidea. *Int. Rev. Ges. Hydrobiol.*, **57**: 257-262.

Fuji, A., 1960. Studies on the biology of the sea urchin. I. Superficial and histological gonadal changes in gametogenic process of two sea urchins, *Strongylocentrotus nudus* and *S. intermedius*. *Bull. Fac. Fish. Hokkaido Univ.*, **11**: 1–14.

Giese, A. C., and J. S. Pearse, 1974. Introduction: general principles. Pages 1–49 in A. C. Giese and J. S. Pearse, Eds., *Reproduction of Marine Invertebrates, Vol. I.* Academic Press, New York.

Gonor, J. J., 1973a. Reproductive cycles in Oregon populations of the echinoid, *Strongylocentrotus purpuratus* (Stimpson). I. Annual gonad growth and ovarian gametogenic cycles. *J. Exp. Mar. Biol. Ecol.*, **12**: 45–64.

Gonor, J. J., 1973b. Reproductive cycles in Oregon populations of the echinoid, *Strongylocentrotus purpuratus* (Stimpson). II. Seasonal changes in oocyte growth and in abundance of gametogenic stages in the ovary. *J. Exp. Mar. Biol. Ecol.*, **12**: 65–78.

Hamann, O., 1888. Die wandernden Urkeimzellen und ihre Reifungsstätten bei den Echinodermen. *Z. Wiss. Zool.*, **46**: 80–98.

Holland, N. D., 1967. Gametogenesis during the annual reproductive cycle in a cidaroid sea urchin (*Stylocidaris affinis*). *Biol. Bull.*, **133**: 578–590.

Holland, N. D., 1971. The fine structure of the ovary of the feather star *Nemaster rubiginosa* (Echinodermata: Crinoidea). *Tissue and Cell*, **3**: 161–175.

Holland, N. D., 1975. Echinodermata: Crinoidea. *In press* in A. C. Giese and J. S. Pearse, Eds., *Reproduction of Marine Invertebrates, Vol. IV.* Academic Press, New York.

Holland, N. D., and A. C. Giese, 1965. An autoradiographic investigation of the gonads of the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.*, **128**: 241–258.

Holland, N. D., and L. Z. Holland, 1969. Annual cycles in germinal and non-germinal cell populations in the gonads of the sea urchin *Psammechinus microtuberculatus*. *Pubbl. Staz. Zool. Napoli*, **37**: 394–404.

Holland, N. D., and Å. Jespersen, 1973. The fine structure of the fertilization membrane of the feather star *Comanthus japonica* (Echinodermata: Crinoidea). *Tissue and Cell*, **5**: 209–214.

Holland, N. D., and S. M. A. Nimitz, 1964. An autoradiographic and histochemical investigation of the gut mucopolysaccharides of the purple sea urchin (*Strongylocentrotus pupuratus*). *Biol. Bull.*, **127**: 280–293.

Holland, N. D., and J. A. Rommel, 1969. A frequency polygon study of oogenesis in the sea urchin *Psammechinus microtuberculatus*. *Pubbl. Staz. Zool. Napoli*, **37**: 657–659.

McPherson, B. F., 1969. Studies on the biology of the tropical sea urchins, *Echinometra lucunter* and *Echinometra viridis*. *Bull. Mar. Sci.*, **19**: 194–213.

Patent, D. H., 1969. The reproductive cycle of *Gorgonocephalus caryi* (Echinodermata; Ophiuroidea). *Biol. Bull.*, **136**: 241–252.

Pearse, A. G. E., 1960. *Histochemistry, Theoretical and Applied* [2nd ed.]. Churchill, London, 998 pp.

Pearse, J. S., 1965. Reproductive periodicities in several contrasting populations of *Odontaster validus* Koehler, a common antarctic asteroid. Biology of the Antarctic Seas. II. *Antarctic Res. Ser.*, **5**: 39–85.

Pearse, J. S., 1969a. Reproductive periodicities of Indo-Pacific invertebrates in the Gulf of Suez. I. The echinoids *Prioncidaris baculosa* (Lamarck) and *Lovenia elongata* (Gray). *Bull. Mar. Sci.*, **19**: 323–350.

Pearse, J. S., 1969b. Reproductive periodicities of Indo-Pacific invertebrates in the Gulf of Suez. II. The echinoid *Echinometra mathaei* (de Blainville). *Bull. Mar. Sci.*, **19**: 580–613.

Pearse, J. S., 1970. Reproductive periodicities of Indo-Pacific invertebrates in the Gulf of Suez. III. The echinoid *Diadema setosum* (Leske). *Bull. Mar. Sci.*, **20**: 697–720.

Pearse, J. S., and B. F. Phillips, 1968. Continuous reproduction in the Indo-Pacific sea urchin *Echinometra mathaei* at Rottnest Island, Western Australia. *Australian J. Marine Freshwater Res.*, **19**: 161–172.

RHOADES, M. M., 1961. Meiosis. Pages 1–75 in J. Brachet and A. E. Mirsky, Eds., *The Cell, Biochemistry, Physiology, Morphology, Vol. III.* Academic Press, New York.

SCHLIEPER, C., 1957. Comparative study of *Asterias rubens* and *Mytilus edulis* from the North Sea (30 per 1,000 S) and the western Baltic Sea (15 per 1,000 S). *Année Biol.,* **33**: 117-127.

SOKAL, R. R., AND F. J. RHOLF, 1969. *Biometry.* W. H. Freeman and Co., San Francisco, 776 pp.

SPICER, S. S., 1963. Histochemical differentiation of mammalian mucopolysaccharides. *Ann. New York Acad. Sci.,* **106**: 379–388.

TANAKA, Y., 1958. Seasonal changes occurring in the gonad of *Stichopus japonicus. Bull. Fac. Fish. Hokkaido Univ.,* **9**: 29–36.

URBANI, E., 1955. Studi comparativi sulla vitellogenesi e sui nuclei vitellini. *Pubbl. Staz. Zool. Napoli,* **26**: 63–109.

VANNINI, E., 1953. Osservazioni sull'accrescimento del nucleolo negli ovociti di *Antedon rosacea. Pubbl. Staz. Zool. Napoli,* **24**: 292–331.

YOSHIDA, M., 1952. Some observations on the maturation of the sea urchin, *Diadema setosum. Annot. Zool. Japonensis,* **25**: 265–271.

Reference: *Biol. Bull.*, **148**: 243–258. (April, 1975)

A COMPARATIVE STUDY OF THE HARD CLAM GONAD DEVELOPMENTAL CYCLE[1]

RICHARD T. KECK, DON MAURER, AND HENRY LIND

University of Delaware, College of Marine Studies, Field Station, Lewes, Delaware 19958

The reproductive physiology of molluscs is of special interest due to their importance as food for man. A thorough knowledge of reproductive cycles is necessary for predicting annual recruitment, interpreting growth, mortality, and survival data, and in the mariculture of these species. A number of studies on the gonadal development of marine invertebrates have been conducted (Giese, 1959). Ansell (1963, 1964, 1968), Ansell and Loosmore (1963), Ansell, Lander, Coughlan, and Loosmore (1964), Ansell, Loosmore, and Lander, (1964), and Ansell and Lander (1967) performed extensive work on the reproduction, spawning, and growth of the hard clam, *Mercenaria mercenaria* (Linne), in England. Shaw (1964, 1965) and Ropes and Stickney (1965) characterized the gonadal cycle of the soft clam, *Mya arenaria* (Linne). Calabrese (1970) described the developmental cycle in the coot clam, *Mulinia lateralis* (Say), in Long Island Sound.

A study of the gonadal cycle of *Mercenaria mercenaria* was undertaken as a subproject of a comprehensive resource survey in Delaware Bay to determine if spawning was occurring and to what degree, to ascertain whether male and female cycles were synchronized, and to compare reproductive cycles of clams from different geographic regions as evidence for physiological races. Loosanoff (1937a) and Porter (1964) studied the gonadal cycles of hard clams in Long Island Sound and North Carolina, respectively. Environmental differences produced different physiological responses in respect to timing of development and developmental pattern. Porter (1964) suggested that the differences were a possible expression of physiological races caused by phenotypic response to environmental factors. This phenomenon is well known in marine organisms and is particularly well documented in the American oyster (Galtsoff, 1964).

MATERIALS AND METHODS

Twenty hard clams were collected monthly for 34 months (January 1971 through October 1973) from each of two areas in Delaware Bay. Area 1, referred to as Delaware Bay, is located approximately 2.7 km northeast of the mouth of Roosevelt Inlet (Coast and Geodetic Survey 1218). The area is characterized by a soft mud (40–80% silt-clay) bottom and is approximately 3–4.5 m deep at mean low water. The clams in this area were obtained by dredging with a standard oyster scrape. Area 2, the Henlopen intertidal flat, is located near the mouth of Delaware Bay inside Cape Henlopen. The area is characterized by a hard sand bottom (5–10% silt-clay). These clams were obtained by hand-raking during periods of low tide. All clams were returned to the laboratory and stored dry in a cool room at approximately 15° C. In all cases tissue samples were removed within 24 hours of the collection in the field.

---

[1] Contribution No. 96, University of Delaware, College of Marine Studies.

Water temperatures in these areas are influenced by both season and tide. During the summer months, temperature falls on the flood tide and rises on the ebb tide. This condition is reversed in winter when the average change during each tidal cycle is approximately 3° C (DeWitt, 1968). Mean monthly water temperatures for lower Delaware Bay with ranges excluding those extremes that occur in less than 1% of the data compiled from Brower, Sisk, and Quayle (1972) appear in Figure 1. Temperature data from S. G. Landers (personal communication, National Marine Fisheries Service Laboratory, Milford, Connecticut) and Porter (1946) are included in Figure 1 to contrast the different physical regimes in the areas for which gonadal data are compared later in the text.

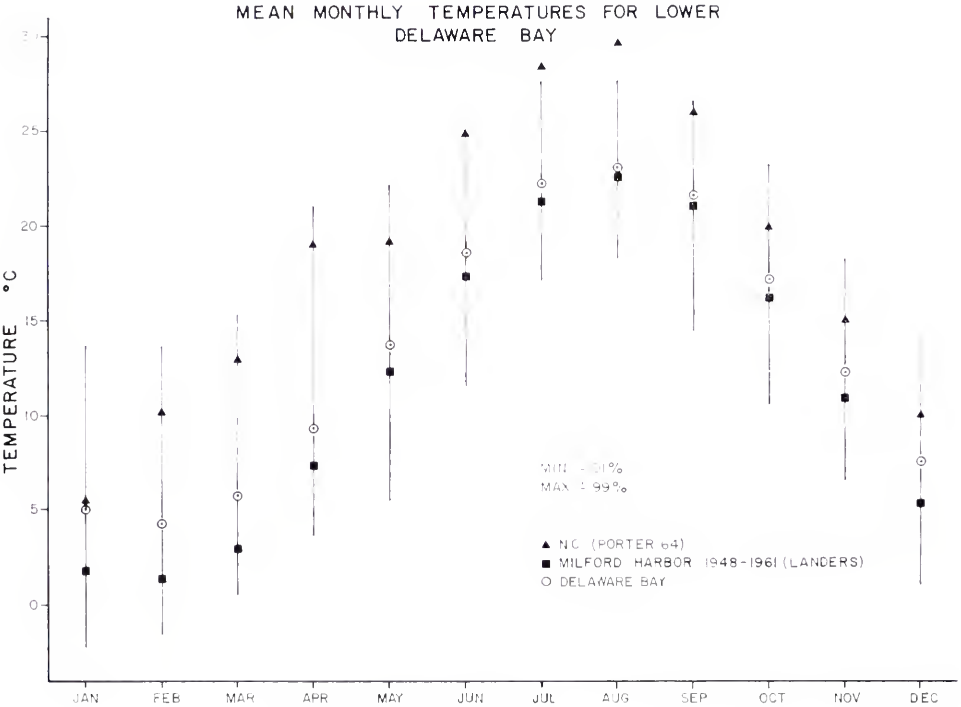


FIGURE 1. Mean monthly temperatures for lower Delaware Bay, North Carolina, and Long Island Sound.

A 1 cm² sample of mantle, gonad, and underlying digestive gland was removed from each of the 20 clams and placed in Bouin's fixative for at least 48 hours. The tissue samples were removed from the area located at the midpoint of a horizontal line connecting the anterior and posterior adductor muscles. Tissues were then prepared for cutting by dehydration in alcohol, clearing in xylene, and embedding in paraffin (Humason, 1967). Gonadal tissue was sectioned at 10 μm and stained with Harris' or Delafield's hematoxylin and counter-stained with eosin Y. Sections were examined under a light microscope and classified by the developmental stage.

To quantify the stage of development of the gonads, the size and number of ova in 20 random sections of tissue 0.2 mm^2 on each female slide were measured and counted. Although the size of an egg depends on the level at which it is cut, the average size based on large numbers of measurements accurately indicates stage of development. The point area method was used to analyze male slides (Ivantsch, 1970). Male gonads were projected onto graph paper and traced to determine the percentage of the lumen filled with radiating bands of spermatids or spermatozoa.

Photomicrographs of representative stages of gametogenesis were taken with a light microscope at × 100 magnification using a standard camera adapter and Nikormat camera. A fine grain, high contrast film, Panatomic X, ASA32, was used.

RESULTS

Developmental stages of the male

The following description of the male and female developmental stages is based upon our observations and criteria compiled from Ropes and Stickney (1965), Porter (1964), and Loosanoff (1937a). The individual stages define qualitative criteria describing the continuous process of change occurring in cells and tissues during the maturation of gonad. Because the terminology for the indifferent phase presents semantic problems, a brief definition for both male and female stages is as follows: although the term indifferent has been applied to specimens that could not be sexually differentiated, it also implies low levels of either spermiogenic or ovogenic acitivity with correspondingly low numbers of recognizable sex cells. The authors found no specimens where it was impossible to determine sex. Basic follicular differences appear to allow differentiation between sexes without the presence of mature sex cells. However, because inactive implies a static condition where absolutely no morphological or biochemical activity is proceeding, the term indifferent appears to be more biologically appropriate to describe that stage occurring between spawned and onset of new development.

Indifferent or inactive stage. The follicle was usually expanded, and only rarely compressed. The basal membrane and follicular cells are dominant and follicular cells often filled the lumen surrounding pycnotic or aberrant cells. The presence of these cells may indicate various forms of structural disintegration. The nuclear boundary is obliterated and the chromatin contracts into deeply stained irregular masses. The remainder of the cell may lose its staining capacity. When the follicle was expanded, there were few, if any, spermatogonia or spermatocytes at the periphery of the lumen. In some cases, the expanded lumen contained spermatozoa in random arrangement and of varying density (Fig. 2A).

Developing or active stage. This phase included the entire process of spermatogenesis. The follicle in this and succeeding stages was expanded. The basal membrane and its attached follicular cells become less apparent as development proceeds. Numerous spermatogonia were present in early stages near the periphery of the lumen. As development proceeded, spermatocytes and spermatids were

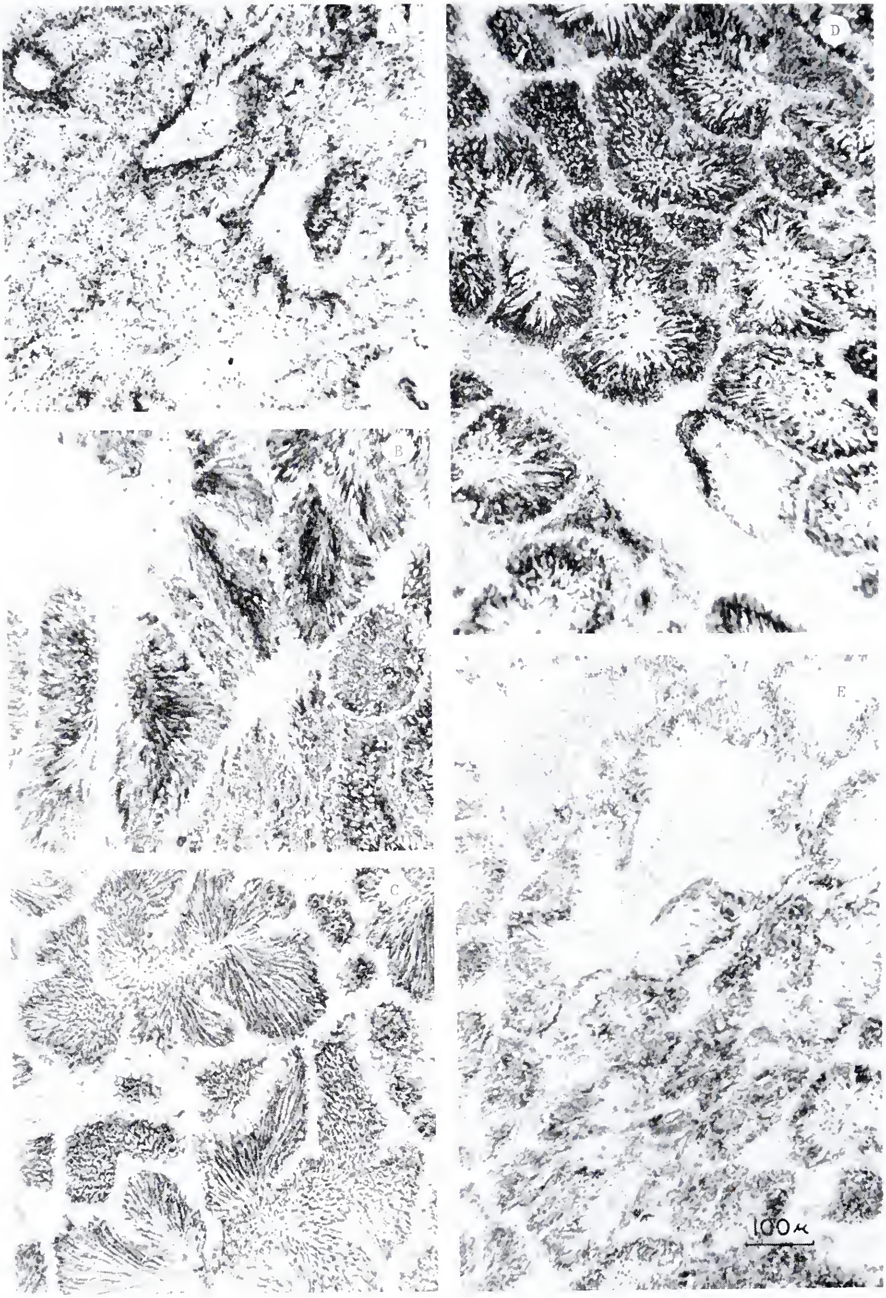


FIGURE 2. Male hard clam developmental stages: (A) indifferent; (B) developing; (C) ripe; (D) partially spawned; (E) totally spawned.

predominant making it difficult to see follicular cells or spermatogonia. Toward the center of the lumen, spermatids differentiated into spermatozoa arranged in dense radiating bands (Fig. 2B).

Ripe stage. The lumen was filled with dense radiating bands of spermatozoa. In fully ripe individuals approximately 95% of the lumen was filled with spermatozoa. The spermatocyte and spermatid layers were thinner than in the developing stage. The spermatid band was usually thicker than the spermatocyte band. However, in extremely ripe individuals the spermatid band was difficult to see due to staining similarities of spermatids and spermatozoa and the overwhelming abundance of mature spermatozoa (Fig. 2C).

Spawned stage. In this stage, consisting of a partially spawned or totally spawned or spent state, the spermatogenic layer (spermatocytes and spermatids) was extremely thin or nonexistent. The lumen had fewer spermatozoa than the ripe stage. The remaining spermatozoa remained in radiating bands. In totally spawned individuals the empty lumen contained few sex cells. The remaining spermatozoa were found near the edge of the follicle, and different amounts of spermatozoa were randomly arranged in the center of the lumen (Figs. 2D and 2E).

Developmental stages of the female

Indifferent or inactive stage. Lumina were semi-contracted and contained unspawned ovocytes. Extremely small ovocytes were still embedded in the follicle wall and recognizable due to the staining of the large basophilic nucleus. This stage was practically nonexistent in the female cycle because partially spawned or totally spawned individuals start ovogenic activity immediately (Fig. 3E).

Developing or active stage. The developing stage was characterized by an increase in the number of ovocytes and size of existing ovocytes. The degree of development was determined by counting and measuring ovocytes. During early development, the ovocytes ranged in diameter between 20–30 μm; as maturation approached, ovocytes varied between 40–50 μm in diameter. The developmental stage was also characterized by thick follicular walls and cytoplasmic inclusions which may be nutritive in function. Small follicles, especially near the periphery of the gonadal mass, frequently were filled with follicular cells. Although in later stages of development many mature or ripe ovocytes existed, there was continuing ovogenic avtivity as shown by the presence of small ovocytes near the periphery of the follicle. Partition cells were often seen during this stage and were also commonly found near the edges of the gonadal mass (Figs. 3A, 3B, and 3D).

Ripe stage. The ripe stage, characterized by large numbers of large mature ovocytes which were more numerous than less developed ovocytes, were generally between 50 and 60 μm in diameter. The follicular wall was extremely thin and expanded and ovogenic activity had practically ceased. The basal attachment of the mature ovocytes was less evident and most ovocytes appeared to be free within the follicular lumen (Fig. 3C).

Spawned stage. The spawned stage consisted of two substages, partially and totally spawned. Totally spawned females were rare. The lumen of the ovarian follicle generally contained a few ripe ovocytes. The follicular wall was semi-

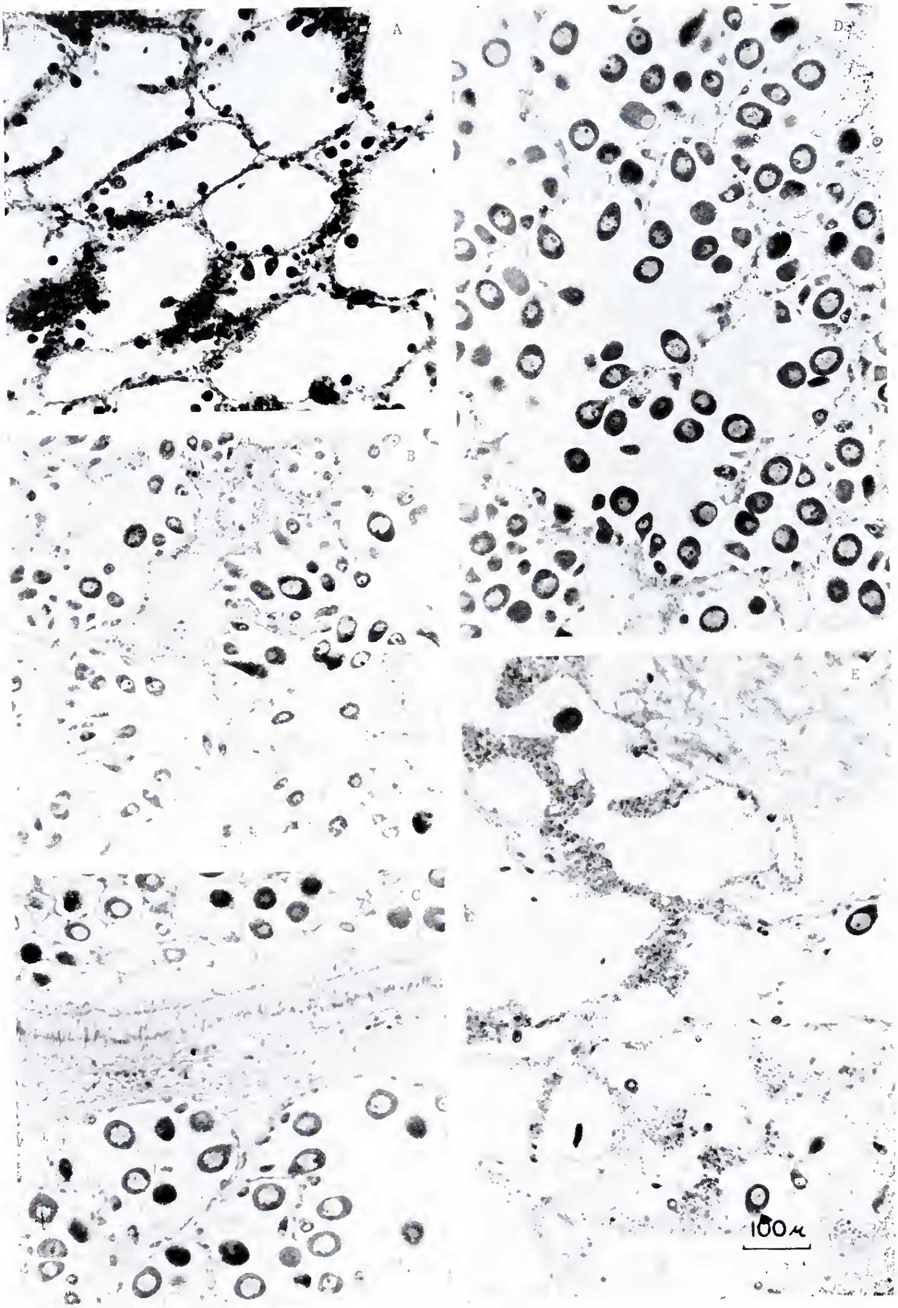


FIGURE 3. Female hard clam developmental stages: (A) developing; (B) developing; (C) partition cells; (D) ripe; (E) spawned, indifferent.

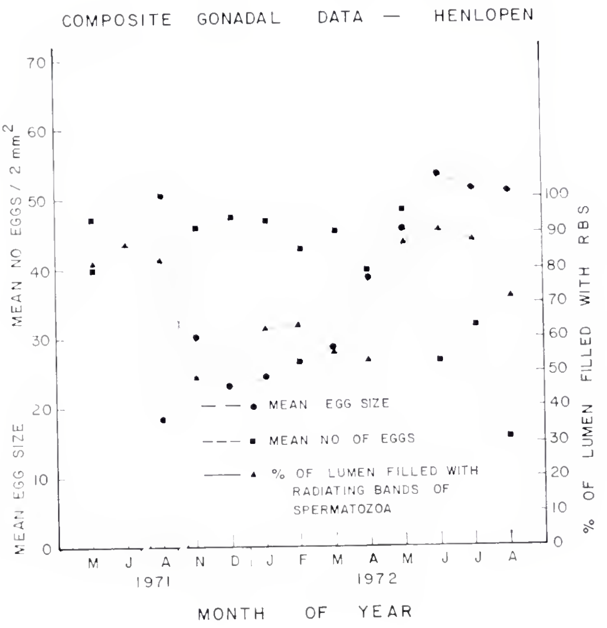
contracted, and thickening may have begun. Ovogenic activity was low, but evidence of regeneration was usually apparent in spawned individuals (Fig. 3E).

Seasonal variation of the gonadal cycle

Quantitative data describing the male and female developmental cycles appear in Figures 4 and 5. The male and female cycles were in phase for both Delaware Bay and Henlopen clams. The percentage of lumina filled with radiating bands of spermatozoa and egg size of females coincided. Peak spawning occurred in the month of August. In the male, peak spawning activity was marked by the beginning of a precipitous decline in percentage of lumina filled with radiating bands of spermatozoa. In the female, heavy spawning activity was represented by the greatest difference between egg size (large) and egg number (low). Delaware Bay clams showed that increases in egg size and egg number were parallel until May. After May, these data diverged, indicating that partial spawning occurred from June to October. The egg number (data) for Delaware Bay clams showed that regeneration started in early August and that ovogenic activity continued slowly toward maturity in May. Henlopen clams showed a sinusoidal pattern in which egg size and egg number shifted. This pattern appears natural in that large egg size indicates there should be fewer eggs per given unit of tissue and small egg size denotes the converse. This phenomenon occurs as the eggs ripen and the follicular lumina expand. The shift usually occurred in May and October, which again reflects the length of the spawning season. The shift also indicated a more rapid regeneration. The Henlopen clams produced the majority of their new ovocytes between September and December. From December to May the egg number was relatively constant with a gradual increase in size reflecting maturation. Comparison of egg number per unit area for Figures 4 and 5 showed that Henlopen clams produced more eggs per unit area, while Delaware Bay clams generally produced larger eggs, representing a possible difference in reproductive potential.

A three-year composite of qualitative observations reflecting all developmental data collected per month for January 1971 through October 1973 is presented in Figures 6 and 7. Data are represented as percentage of individuals in each developmental stage per month. The data show that there was no significant difference in developmental pattern between Delaware Bay and Henlopen clams. However, males from both Delaware Bay and Henlopen seemed to lag behind females until late spring when rapid spermatogenic activity brought the male and female cycles into phase. The lag is probably a result of the fact that many male clams spend a period of time in the indifferent or the inactive stage (fall and winter months), while the females start regeneration immediately.

The qualitative and quantitative data support a similar developmental trend. Spawning activity commenced in June and continued until October where some individual appeared totally spent. Peak spawning activity for both males and females occurred in August and September. Females started ovogenic activity immediately, as early as July and August for partial spawners. A significant percentage of the males became indifferent after spawning in October (Henlopen mean, 40%; Delaware Bay mean, 18%). This percentage of indifferent males remained relatively constant until May when rapid development occurred.



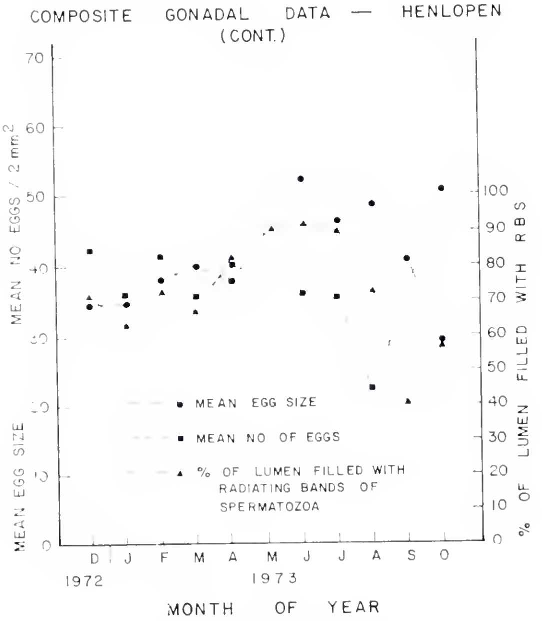


FIGURE 4. Quantitative gonadal data, Henlopen.

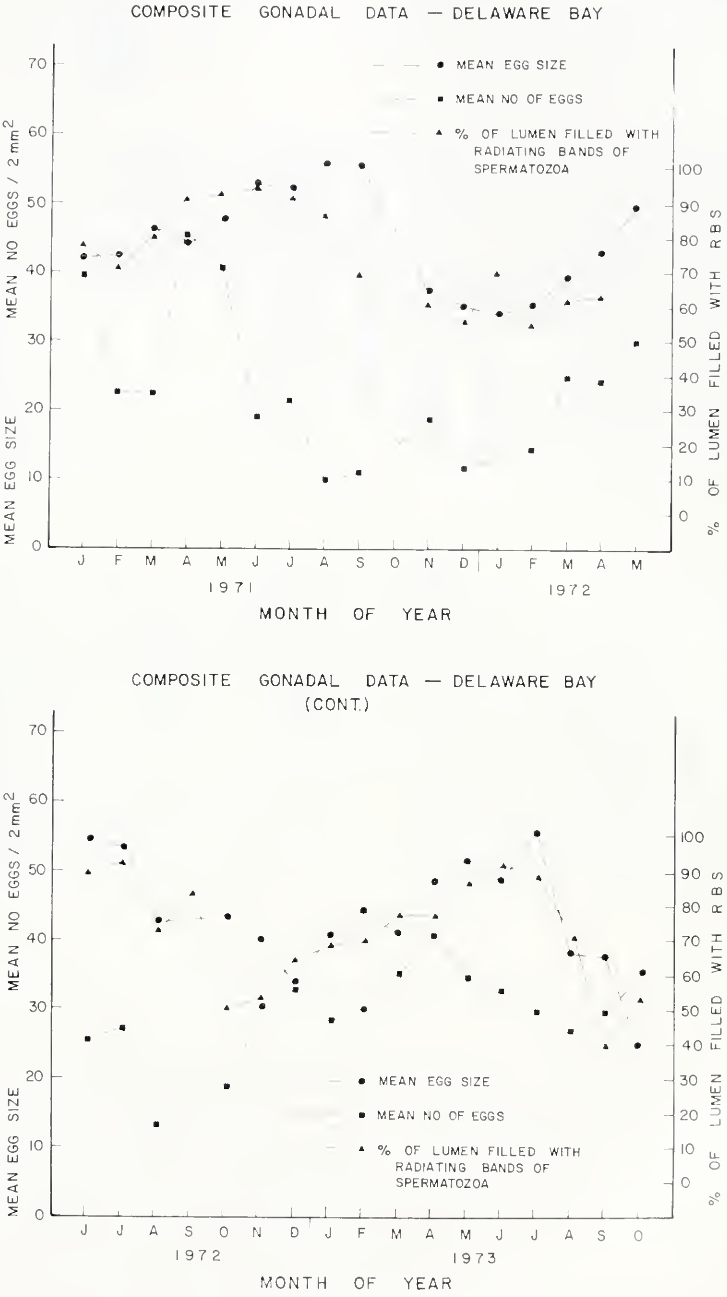


Figure 5. Quantitative gonadal data, Delaware Bay.

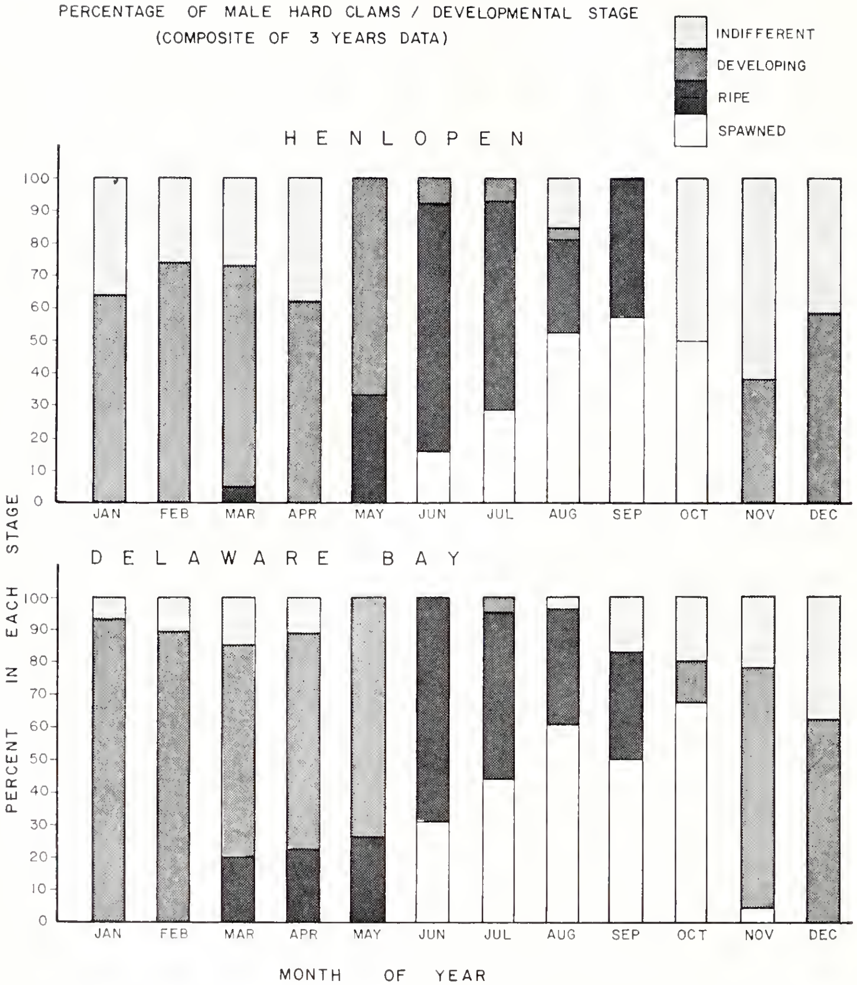


FIGURE 6. Percentage of male hard clams/developmental stage (qualitative criteria).

Analysis of the female developing stage requires consideration of both qualitative and quantitative data. Developmental stages in Henlopen and Delaware Bay females indicated a similar pattern. However, although both groups were in the developing stage, the mechanism was different. Henlopen clams regenerated large numbers of ovocytes rapidly, which slowly increased in size during the winter and spring. Delaware Bay clams developed slowly in both size and number of ova for a long period of time. Ripe individuals appeared as early as December (10% of individuals). From January through April, there was a slight increase in the percentage of ripe individuals per month. Between April and May, rapid ovogenic activity produced large numbers of ripe individuals. June was characterized by the greatest percentage of ripe individuals and the onset of spawning activity for both males and females.

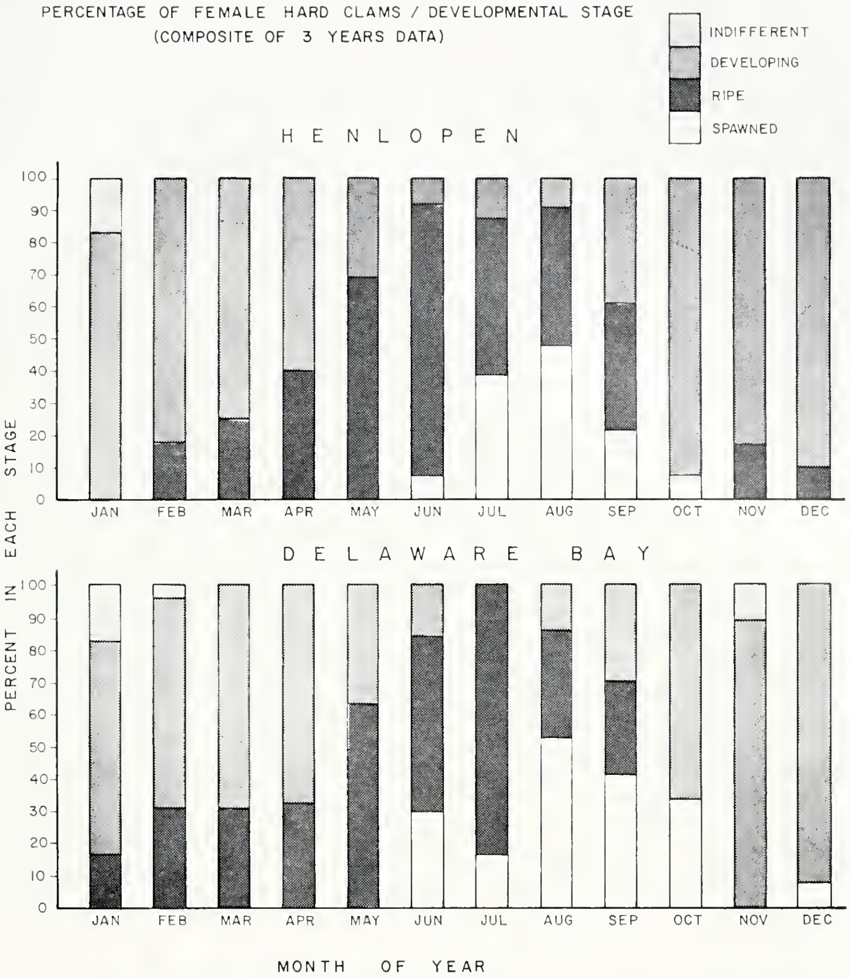


Figure 7. Percentage of female hard clams/developmental stage (qualitative criteria).

Discussion

The data presented here provide some similarities and differences to the work of Loosanoff (1937a) and Porter (1964). Temperature is an important factor affecting the regulation of the gonadal cycle in a variety of marine bivalves (Galtsoff, 1964; Loosanoff, 1937a, 1937b; Landers, 1954; Carriker, 1961; Ansell, Lander, Coughlan, and Loosmore, 1964; Calabrese, 1970; Giese, 1959). The temperature and salinity regimes which exist in Long Island Sound, Delaware, and North Carolina are different. However, mean water temperatures in Delaware more closely resemble mean temperatures in Long Island Sound than those in North Carolina (Fig. 1). Local conditions can drastically affect the gonadal cycle. Ansell, Lander, Coughlan, and Loosmore (1964) and Tinsman (1973) have shown that thermal effluent produced premature ripening of gonads in hard clams and oysters, respectively. Carriker (1961) showed that depth of water and circulation together with temperature greatly affect the onset of spawning activity in hard clams. H. G. Lind (personal communication, University of Delaware) showed that temperatures in a shallow Massachusetts salt pond caused two spawning periods in the soft clam, *Mya arenaria.* There are normally two spawning periods south and one north of Cape Cod for soft clams in estuarine waters (Ropes and Stickney, 1965). Landers (1954) suggested that intertidal clams spawned earlier than those from deeper water. Keck, Maurer, Daisey, and Sterling (1973) reported this phenomenon for oysters in tributaries of the Delaware Bay.

Table I provides a comparison of monthly developmental differences among bivalves studied by Loosanoff (1937a), Porter (1964), and those under present investigation. The spawning period, June to October, was similar to that reported by Porter (1964) and the present study. Loosanoff noted that spawning did not occur until late July in Long Island waters. Spawning in males was more complete than in females in all three areas. The proportion of totally spawned individuals of both sexes increased from south to north. R. W. Menzel (personal communication, Florida State University) states that field observations indicated that *M. mercenaria* and *M. campechiensis* complete spawning as early as April in Florida waters and are much more difficult to condition than northern clams. This appears to be linked to the greater variability of summer temperatures in northern waters. The rate of temperature change probably provides a stronger spawning stimulus than absolute temperature. Normal spawning temperatures are slightly different in the three regions (27–30° C in North Carolina, Porter, 1964; 25–27° C in Delaware, and 23–25° C in Long Island, Loosanoff, 1937a). Regeneration of gonadal tissue was evident as early as June in North Carolina waters where Porter observed that a "late ripeness" and capability to respawn were evident. Although regeneration was evident in August in Delaware bivalves, the restoration process required a longer refractory period. Clams that showed a high degree of ovogenic activity after partial spawning were those that appeared ripe in late fall and early winter. Loosanoff (1937a) reported that regeneration also starts immediately after spawning in Long Island waters, that is, during September and October.

Extrusion of unspent ova remains an unexplained phenomenon in this study. Loosanoff (1937a, 1937b) reported that the majority of unspent ova were extruded in a normal manner by November. Porter (1964) reported that at least 50% of his clams retained a ripe appearance through the fall and winter. Major extrusion occurred during February or March. In the present study, there was insufficient information to determine the time of extrusion. However, throughout the late fall, winter, and springs, partition cells appeared near the periphery of the gonadal mass in mature individuals. Porter (1964) suggested that cytological destruction may occur within the partition cells. Quite possibly, as suggested by Ansell (1961), the older ovocytes were carried over in partition cells and redeveloped follicles. As noted by Loosanoff (1937a), the more complete the spawning, the more rapid regeneration begins.

The pattern and timing of development differ widely among clams from different geographic areas. Porter (1964) reported major primary redevelopment in

INE LIBRARY

TABLE I

Comparison of the sequence of hard clam gonadal development in three geographic regions

Comparative developmental stage	North Carolina (Porter, 1964)	Delaware (present study)	Long Island (Loosanoff, 1937a)
Partial spawning ♀ + ♂	June	June	July
Maximum spawning	Aug. (27–30° C)	Aug. (25–27° C)	Aug. (23–25° C)
Length of spawning season ♀ + ♂	June–Sept.	June–Sept.	July–Aug.
Regeneration ♀	[early (summer) or late (winter)]	Oct.–Dec.	Oct.–Dec.
Percent remaining ripe + timing of extrusion	50% through Dec.	Fate of unspent ova undetermined; partition cells—evident Nov.–March	Extrusion complete by Nov.
Percent of individuals in an indifferent stage ♀ + ♂	60–90%	Approximately 40% of males—less than 10% females	No indifferent stage
Major period of development ♀	Dec.–Jan.	Long, slow process; Oct.–March	Long, slow process; Oct.–March
Major period of rapid gametogenic activity ♀ + ♂	March	April	May
Presence of follicular cells	Present in developing stages	Prominent in developing stages	Absent
Majority of individuals in ripe stage ♀ + ♂	May	May and June	June

December and January with secondary activity in March. Loosanoff (1937a, 1937b) described rapid redevelopment in October through November. December through April marks a slowed period of continued maturation.

The present study provided two divergent patterns of development. The Henlopen clams developed very similarly to those discussed by Loosanoff (1937a), but preceding that timetable by approximately one month. The Delaware Bay clams provided a pattern different from that reported by both Porter and Loosanoff. In 1971–1972 (Fig. 4), female redevelopment started late in November and then continued with a slow increase in size and number of eggs. In 1972–1973, the process started earlier, but the slow steadily increasing trend was again apparent. Henlopen clams are in close proximity to cooler oceanic water in the summer and warmer oceanic water in the fall and winter, and this may be responsible for the difference in developmental pattern. Male clams from both Delaware Bay and Henlopen developed similarly to those discussed by Porter. However, a smaller percentage of clams in Delaware waters were found in the indifferent stage. Loosanoff did not report any indifferent stage for Long Island male clams. Vigorous spermatogenic activity occurred in March (North Carolina), May (Delaware), and June (Long Island). In addition, Long Island clams showed more advanced spermatogenic activity in the fall than either Delaware or North Carolina clams.

The presence of follicular cells of North Carolina clams and their absence in Long Island clams was a major difference noted by Porter (1964). Both Delaware and Henlopen clams contained follicular cells. These vacuolated cells, as reported by Porter (1964), appeared in small follicles near the periphery of the gonadal mass. These cells were evident in all tissues categorized as developing (fall through spring). Follicular cells were reported by Ansell (1961) in *Venus striatula* and Ropes and Stickney (1962) in *Mya arenaria*. Various functions such as nutrition or phagocytosis have been ascribed to the cells. Because of their location in small follicles, it is likely they have a function in expansion of the developing follicle.

The study of the hard clam gonadal cycle in Delaware Bay provides data from an intermediate location to compare with Loosanoff (1937a) and Porter (1964). The data collected by Loosanoff and Porter had the advantage of large number of individuals during a one-year period. The present study had fewer samples/year, but the data were collected over a three-year period and provide almost complete coverage through several cycles.

The clams in Delaware Bay show gonadal development intermediate between Long Island and North Carolina clams. Also, clams from essentially the same area (Delaware Bay and Henlopen tidal flats) displayed divergent developmental patterns. These different gonadal patterns are due to environmental differences. Loosanoff commented that the sexual cycle of the hard clam was not in phase with other bivalve molluscs. The major period of development was in the fall as water temperatures decreased. Recent unpublished studies by the authors suggest that the glycogen cycle for the clam is widely different from that of the oyster. A spent oyster is devoid of sex products and generally in poor condition. The hard clam retains a relatively high proportion of glycogen or condition index even after spawning. The maintenance of a high condition level allows immediate gonadal redevelopment in the fall.

SUMMARY

1. This study has shown that hard clam reproductive cycles in Delaware Bay are in phase and that spawning activity during 1971–1973 was of sufficient intensity to provide ample larval stocks.

2. The gonad developmental patterns for clams in Delaware are intermediate between those for Long Island and North Carolina. The data provide supporting evidence that different physiological races exist in the three areas compared. A further test on the validity of physiological races could be determined by studying the developmental patterns of Long Island and North Carolina clams held experimentally in Delaware Bay.

3. Environmental factors attribute to subtle differences in reproductive physiology as evidenced by the different mechanisms of regeneration and development between Delaware Bay and Henlopen hard clam females.

The authors wish to thank Dr. Melbourne R. Carriker, Dr. R. W. Menzel, Dr. L. Watling, Mr. Hugh Porter for critical review of the manuscript, and Dr. Jonathan Taylor and Ms. Ann Taylor who provided photographic support for the project. This study was supported in part by the National Marine Fisheries Service, P. L. 88–309, Project No. 3–135R.

LITERATURE CITED

ANSELL, A. D., 1961. Reproduction, growth, and mortality of *Venus striatula* in James Bay, Millport. *J. Mar. Biol. Ass. U.K.*, **41**: 191–215.

ANSELL, A. D., 1963. *Venus mercenaria* (L.) in South Hampton water. *Ecology*, **44**: 396–397.

ANSELL, A. D., 1964. Some parameters of growth of mature *Venus mercenaria* L. *J. Cons. Cons. Int. Explor. Mer.*, **29**: 214–220.

ANSELL, A. D., 1968. The rate of growth of the hard clam, *Mercenaria mercenaria* (L.), throughout the geographical range. *J. Cons. Cons. Int. Explor. Mer.*, **31**: 364–409.

ANSELL, A. D., AND F. A. LOOSMORE, 1963. Preliminary observations on the relationship between growth, spawning, and condition in experimental colonies of *Venus mercenaria* L. *J. Cons. Cons. Int. Explor. Mer.*, **28**: 285–294.

ANSELL, A. D., K. F. LANDER, J. COUGHLAN, AND F. A. LOOSMORE, 1964. Studies on the hard shell clam, *Venus mercenaria*, in British waters. I. Growth and reproduction in natural and experimental colonies. *J. Appl. Ecol.*, **1**(1): 63–82.

ANSELL, A. D., F. A. LOOSMORE, AND K. F. LANDER, 1964. Studies on the hard shell clam, *Venus mercenaria*, in British waters. II. Seasonal cycle in biochemical composition. *J. Appl. Ecol.*, **1**(1): 83–95.

ANSELL, A. D. AND K. F. LANDER, 1967. Studies on the hard shell clam, *Venus mercenaria*, in British waters. III. Further observations on the seasonal biochemical composition and on spawning. *J. Appl. Ecol.*, **4**(2): 524–535.

BROWER, W. A., D. D. SISK, AND R. G. QUAYLE, 1972. *Environmental Guide for Seven U. S. Ports and Harbor Approaches.* NOAA Evn. Data Ser. Nat. Climatic Center, Asheville, N. C., 166 pp.

CALABRESE, A., 1970. Reproductive cycle of the coot clam, *Mulinia lateralis* (Say), in Long Island Sound. *Veliger,* **12**(3): 265–269.

CARRIKER, M. R., 1961. Interrelation of functional morphology, behavior, and autecology in early stages of the bivalve, *Mercenaria mercenaria*. *J. Elisha Mitchell Sci. Soc.*, **77**(2): 168–241.

DEWITT, W., 1968. The hydrography of the Broadkill River estuary. *Master's Thesis, University of Delaware,* Newark, Delaware, 89 pp.

GALTSOFF, P. S., 1964. The America oyster, *Crassostrea virginica* Gmelin. *U. S. Fish. Wildl. Serv. Fish. Bull.*, **64**: 1–480.

GIESE, A. C., 1959. Comparative physiology: annual reproduction cycles of marine invertebrates. *Annu. Rev. Physiol.*, **21**: 547–576.

HUMASON, G. L., 1967. *Animal Tissue Techniques.* W. H. Freeman and Company, San Francisco, 569 pp.

IVANTSCH, G. F., 1970. The effects of high temperatures on the gonadal area of representative cross sections of *Mytilus edulis* L. *Master's Thesis, University of Delaware,* Newark, Delaware, 67 pp.

KECK, R. T., D. MAURER, W. DAISEY, AND L. STERLING, 1973. *Annu Rep. 1972–1973 Marine Invertebrate Resources,* 71 pp. Submitted to Dept. Nat. Res. and Env. Cont., Dover, De.

LANDERS, W. S., 1954. Seasonal abundance of clam larvae in Rhode Island waters 1950–1952. *U. S. Fish Wildl. Serv. Spec. Sci. Rep. Fish., No.* **117**: 1–29.

LOOSANOFF, V. L., 1937a. Seasonal gonadal changes of adult clams, *Venus mercenaria* (L.). *Biol. Bull.*, **72**(3): 406–416.

LOOSANOFF, V. L., 1937b. Development of the primary gonad and sexual phases in *Venus mercenaria* Linnaeus. *Biol. Bull.,* **72**(3): 389–405.

Porter, H. J., 1964. Seasonal gonadal changes of adult clams, *Mercenaria mercenaria* (L.) in North Carolina. *Proc. Natl. Shellfish. Ass.*, **55**: 35–52.

Ropes, J. W., and A. P. Stickney, 1965. Reproductive cycle of *Mya arenaria* in New England. *Biol. Bull.*, **128**(2): 315–317.

Shaw, W. N., 1964. Seasonal gonadal changes in female soft shell clams, *Mya arenaria*, in the Tred Avon River, Md. *Proc. Natl. Shellfish. Ass.*, **53**: 121–132.

Shaw, W. N., 1965. Seasonal gonadal cycle of the male soft shell clam, *Mya arenaria*, in Maryland. *U. S. Fish. Wildl. Serv. Spec. Sci. Rep. Fish.*, No. **508**: 1–5.

Tinsman, J. C., 1973. The effects of thermal effluent on the American oyster, *Crassostrea virginica* Gmelin, in Indian River Bay, Delaware. *Master's Thesis, University of Delaware,* Newark, Delaware, 126 pp.

Reference: *Biol. Bull.*, **148**: 259–273. (April, 1975)

COCKROACH MOLTING. I. TEMPORAL ORGANIZATION OF EVENTS DURING MOLTING CYCLE OF *BLATTELLA GERMANICA* (L.)

J. G. KUNKEL

Biology Department, Case-Western Reserve University, and Zoology Department, University of Massachusetts at Amherst, Amherst, Massachusetts 01002

The molting cycle of insects has long been used as a model system for studying development. Studies on hormonal control mechanisms (Wyatt, 1972; Doane, 1973; Willis, 1974), pattern formation (Locke, 1967; Lawrence, 1973) and neuronal development (Edwards, 1969) have effectively used the molting and metamorphic cycles of insects to add to our understanding of development. Two insect species which have contributed greatly to this understanding are the blood-sucking bug, *Rhodnius prolixus* (Wigglesworth, 1934), and the Cecropia silkmoth, *Hyalophora cecropia* (Williams, 1946). One reason both of these insects are particularly suited for studies of development is that their development can be controlled by extrinsic environmental cues (Edwards, 1966) allowing them to be manipulated by an experimenter. This paper introduces an approach to the study of cockroach development using feeding (Kunkel, 1966) and regeneration (O'Farrell and Stock, 1953) as extrinsically controllable cues for regulating the development of cockroach cultures. These cultures are used as sources of animals of specified developmental age for experimentation (Kunkel, 1973, 1975; Kunkel and Lawler, 1974). In particular this paper describes a structuring of the molting cycle of *Blattella germanica* into two functionally and experimentally separable phases, the intermolt phase and the molt phase. I also describe some simple assays for landmark events during the molting cycle and establish some limits of usefulness for the synchronous cultures.

MATERIALS AND METHODS

Culturing the German cockroach, *Blattella germanica,* synchronously by controlling feeding has been described previously (Kunkel, 1966). All cultures in this study were raised at 30° C in convection type incubators. A modification of the basic procedure, which saved considerable time and effort, is to allow the *B. germanica* larvae to go through the first two instars with continuous food availability without storing them in between. Thereafter the larvae are stored at 15° C without food after each ecdysis until the culture is ready to be refed.

The orange body color mutant, *or,* obtained from Dr. Mary Ross of Virginia Polytechnic Institute, allows whole mounts of the epidermis to be made—a practice not practicable using strains with the melanised wild type cuticle. In the *or* strain, the epidermal cells and developing cuticular structures of whole mounts can be visualized using transmitted light and phase contrast microscopy. The Feulgen reaction for DNA was routinely carried out on whole mount material fixed in Bouins fluid. The fourth instar was chosen for making a detailed temporal map of molting cycle processes because it is the last larval instar in *B. germanica* in which there is no significant sexual effect on instar length.

Tissue was fixed, embedded and sectioned for electron microscopy by techniques described in Locke (1966). The tonicity and pH of the fixatives were adjusted to 300 milliosmoles and pH 6.5 for *B. germanica* tissue. Gross histological morphology was studied in 1–2 micron sections of glutaraldehyde, osmium, ethyl gallate fixed material embedded in ester wax (Wigglesworth, 1957).

The processes to be dealt with in this paper can best be defined in terms of their assays. One group of processes can be called quantal events, in that they occur instantaneously or at least quickly enough so that whenever an observation is made on a sample of animals from a synchronous culture, they can be classified into two subsets: the subset, A, in which the event has occurred and the complementary subset, A′, in which the event has not occurred. The proportion, $A/(A' + A)$, of a sample of animals which has passed the event is plotted against time. The time, T_{50}, when 50 per cent of the culture has passed the event can be estimated graphically using probability paper or, more usefully, calculated by probit or logit analysis (Finney, 1952) which also provide a standard error of the T_{50}. The standard error allows a decision to be made on the sequence of the events.

A more continuous process, epidermal cell proliferation, was monitored in two ways. First, epidermal nuclear density (nuclei/unit area) was followed by projecting Feulgen-stained nuclei of the epidermal layer of whole mounts onto a standard area of graph paper by means of a *camera lucida*, tracing the epidermal nuclear outlines and computing nuclear density. Secondly, the spatial-temporal relationship of mitoses was established using colchicine to collect all mitoses at metaphase as they occurred. A preliminary study demonstrated that a dose of 0.02 μg/mg live weight (5×10^{-5} M) causes mitoses to be arrested at metaphase for the next 8 hours after which some chromosomal disintegration becomes evident. With smaller doses bipolar mitoses start appearing before eight hours are up. Higher doses cause substantial chromosomal disintegration before the eighth hour.

RESULTS

Descriptive synopsis of the molting cycle events of B. germanica

The spatial and temporal patterns of events in the abdominal sclerites of segments 2 to 5 are consistent with each other. Looking at any one segment gives a good picture of what is happening in the others. Segments 6 and 7 have a superabundance of dermal glands in their tergite region (Roth and Stahl, 1956) which makes them distinctly different from segments 2–5. Within each segment there is considerable heterogeneity in mitotic behavior from area to area. Figure 1A gives a perspective view of the intertergite-tergite of the fifth abdominal segment. The intertergite is devoid of bristles and dermal glands whereas the tergite region, as visualized in Figure 1B, is pockmarked with evenly-spaced dermal glands and bristles. The high density of organules [a term coined by Lawrence (1966) for small organs such as dermal glands and bristles, which are formed of small groups of cells] in the tergite region makes the analysis of the cell types and cell numbers for the tergite difficult. The intertergite, however, is relatively simple; without the large number of organules, the intertergite is made up of two stratified cell types:

an apical layer of generalized epidermal cells responsible for secreting the cuticle, and a basal layer of oenocytes, which are said to be terminally differentiated derivatives of generalized epidermal cells (Wigglesworth, 1961).

Mitoses start appearing in the epidermis of the abdomen twenty-four hours after feeding. The majority of mitoses which can be collected using colchicine during this early phase of the molting cycle are found in the tergite region. They appear singly or in groups of two and rarely in larger clusters, Figure 1C. They are particularly abundant in the tergite region of segment 6 and 7, the area rich in dermal glands and bristles. Figure 1C also illustrates another feature of these early mitoses: they occur primarily basally, out of the plane of the generalized epidermal cell nuclei in the plane of the relatively larger oenocyte nuclei. The above characteristics identify these early cell divisions as organule type mitoses (Wigglesworth, 1953; Lawrence, 1966). Another type of event occurring at this early time in apical layers of both the tergite and intertergite is cell death (Wigglesworth, 1942) as indicated by pycnotic nuclei and chromosomal debris, Figure 1E. These differentiative processes, organule mitoses and cell death, occur in a period which will be called the intermolt phase. The molting phase is signaled by the appearance of proliferative mitoses (*cf.* Holtzer, Weintraub, Mayne, and Mochan, 1972), mitoses which generate cells which will again produce general cuticle. These mitoses are typified by being evenly distributed, rather than clustered, during colchicine collection, Figure 1D. They also typically occur in the apical plane along with the non-mitotic generalized epidermal cells rather than toward the basal plane of the oenocytes.

After mitoses in the epidermis are no longer seen, a molting space becomes evident. This event called apolysis (Jenkin and Hinton, 1966), is easily seen in whole mounts by looking at the posterior edge of the tergite and particularly at the innervation of the thick walled chemoreceptor (Slifer, 1970) type bristles along this border. In order to maintain innervation of old sensory bristles during the digestion and resorption of the old cuticle, the distal processes of sensory neurons are maintained within a protective cuticular sheath, Figures 2A, 3B, and 3D (Slifer, 1970). This protective sheath issues from the tip of the new bristle and must be of considerable length since it must provide enough slack to allow the shaft of the new bristle to grow out laterally in the molting space, Figures 2C, and 2D, and still maintain innervation of the old bristle by the sensory distal processes. Extrusion of these coiled sheaths into the molting space delineates the space. It is not known whether apolysis itself occurs substantially before the appearance of these coils. The coils are present for a time before any new bristle growth is evident.

While a new bristle shaft is forming in the molting space, Figures 2B-F, a new cuticular sheath is forming about the distal processes in the center of the new bristle shaft, Figure 3A, and extending down deep into the epidermis, Figure 3C, providing a new slack sheath for bristle morphogenesis of the subsequent molting cycle. The end of superficial bristle morphogenesis is preceded by the formation of a ribbing of the bristle shaft seen in phase contrast of whole mounts, Figure 2D, by the appearance of fibrous rods at the periphery of the trichogen cell in electron micrographs, Figure 3A (Lawrence, 1966; Locke, 1966), and appearance of peripheral densities in Osmium-ethyl gallate stained light microscope sections of bristle

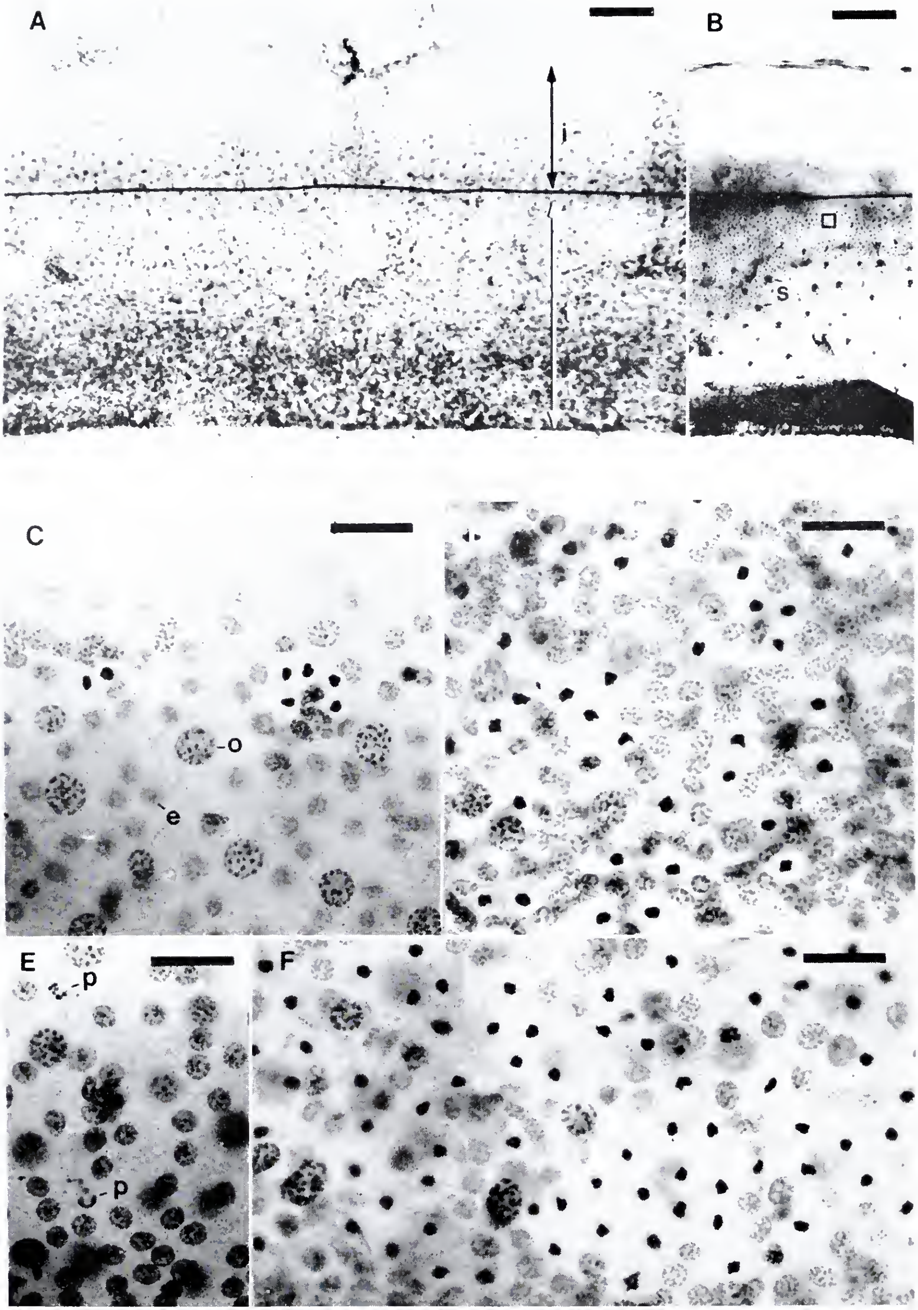


FIGURE 1. Whole mounts of *B. germanica* abdominal sclerites, showing: a) fifth abdominal tergite, **t**, and intertergite, **i**; b) ammoniacal-silver nitrate stained dermal glands (three within square) and bristle sockets, **s**; c) intermolt epidermis with two clusters of colchicine collected

shafts, Figure 2F. The mature bristle shaft prior to ecdysis has a thick cuticle, Figure 2E. During bristle morphogenesis the molting space is well formed, Figure 2F, over the entire epidermis with the exception of the muscle attachments, Figure 2G. The muscle attachments to the old cuticle persist while the old endocuticle is being digested (Lai-Fook, 1967; Caveney, 1969), but release when substantial digestion of the old cuticle is completed, well before the actual time of ecdysis.

Quantalization of developmental processes

Some processes during the molting cycle are innately quantal as defined. However, some, such as bristle morphogenesis, must be artificially quantalized by arbitrarily defining starting or end points which can be treated as quantal events. In some cases such as epidermal proliferation, no satisfactory categorical decision can be made about the beginning of proliferation since it begins in an area of the tergite which is confused with organules. However, a definitive statement can be made about whether proliferation has ended in a given animal. Below are listed a set of operational definitions which can be conveniently applied in cockroaches to set up a temporal map of events during the molting cycle. The first three definitions are of phenomenological events which are important in the control of the molting process and the remaining definitions are morphological events which can be easily assayed in whole mounts or by superficial inspection.

The head critical period is the time in each animal after which the head is no longer necessary to complete a molting cycle. Animals past the head critical period will molt even though their head is ligated from their body.

The prothoracic critical period is the time in each animal after which the prothorax, containing the prothoracic (ecdysial) gland, is no longer necessary to complete a molting cycle. Similar to the above assay, animals past the prothoracic critical period molt even with a ligature between the pro-and meso-thorax.

The regeneration critical period is the time in each animal after which if a leg is autotomized, regeneration of the leg is postponed until the next molt (O'Farrell and Stock, 1953).

Appearance of protein granules in the fat body is indicated by appearance of Millon positive granules in fat body whole mounts at the resolution of the light microscope.

The end of epidermal proliferative mitosis is the time in each animal in which scanning of 2 entire feulgen-stained whole-mounted tergites and intertergites (10^4 cells), Figure 1A, shows no mitotic figures.

Nerve coil appearance in the molting space is the time in each animal after which whole mounts of the epidermis show coils of distal processes of sensory neurons in their cuticular sheath in the molting space, Figure 2A.

The beginning of bristle morphogenesis in the individual is taken as the time after which protrusions from the bristle cells are seen in the molting space at the posterior edge of tergal whole mounts observed under phase contrast. In Figure

---

mitoses, with oenocytes, **o**, generalized epidermal cells, **e**; d) proliferative phase colchicine mitoses; e) feulgen positive pycnotic nuclear debris, **p**; and f) an eight-hour colchicine collection of mitoses during the proliferative phase. The calibration lines represent, in A, B, 100 μ, and in C-E, 25 μ.

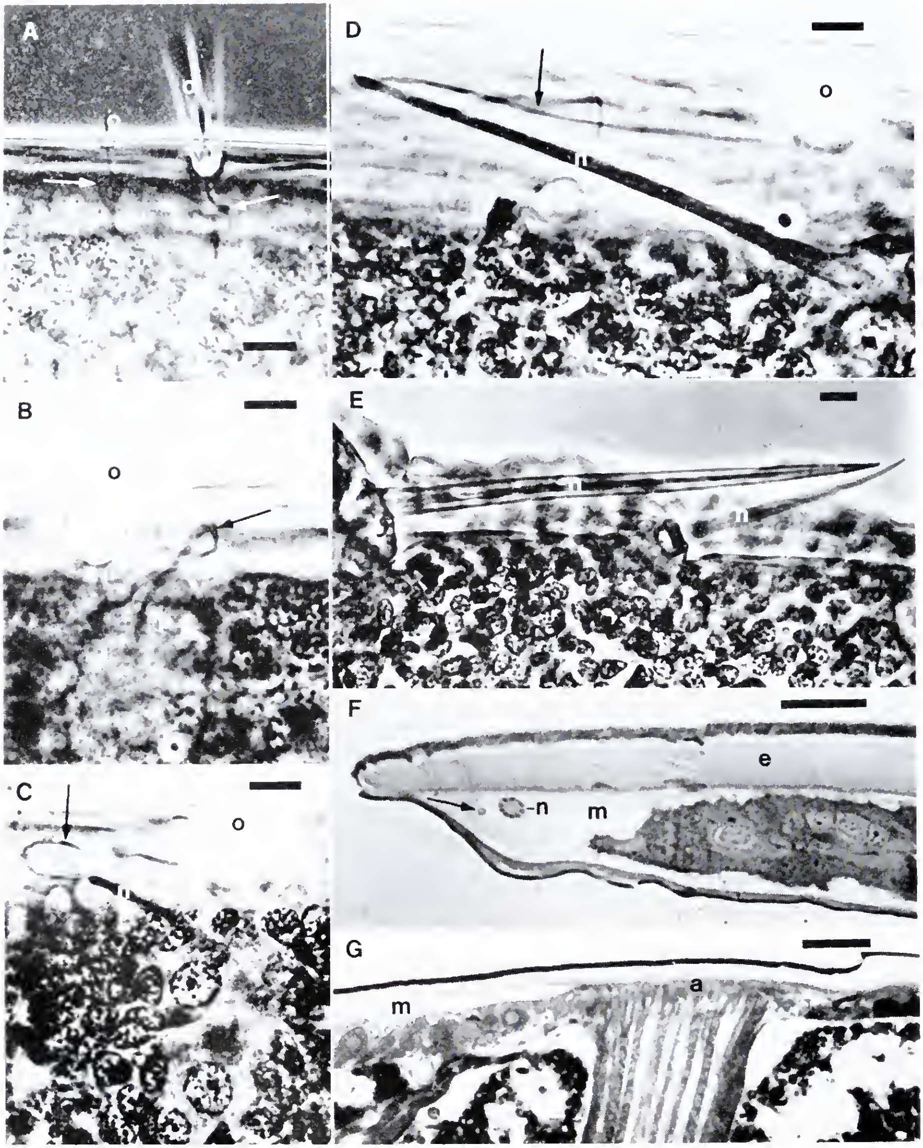


FIGURE 2. Stages in apolysis and bristle replacement. A) Nerve coils appear (arrows) at apolysis. B) A protuberance, **p**, is the first sign of bristle morphogenesis. C) Slack nerve coil is available during new bristle, **n**, growth, showing also trichogen, **tr**, and tormogen, **to**, cell nuclei. D) In the terminal stage of new bristle elongation, the nerve coil maintains its connection with the old bristle, **o**. E) The new bristle shows thick cuticle deposition. F) A longitudinal section cut through the posterior edge of a tergite is shown from a stage equivalent to D above with molting space, **m**, and undigested old endocuticle, **e**. G) Another section is shown from the same animal as F above, but with muscle attachment, **a**. The calibration lines represent in A-E, 10 μ, and in F, G, 50 μ.

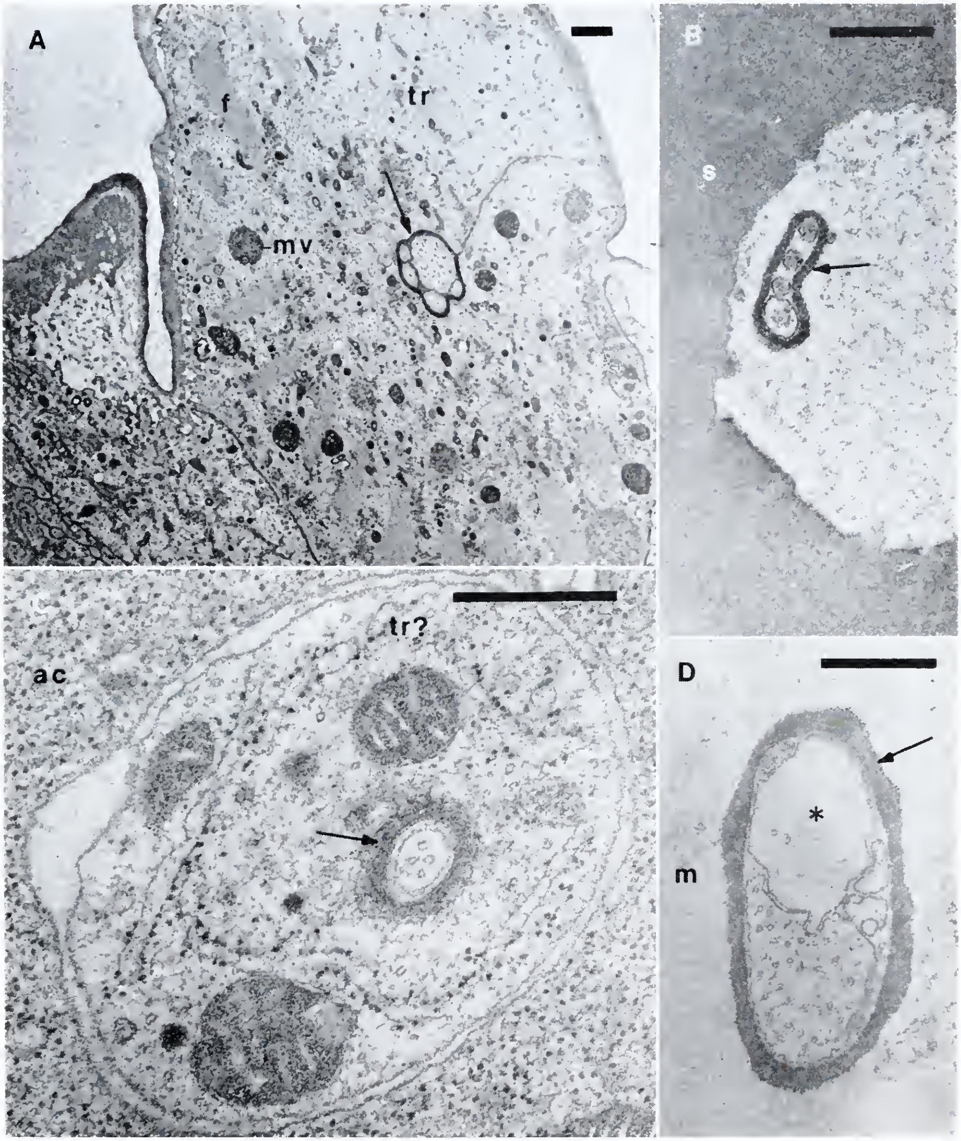


FIGURE 3. Ultrastructural relationships of nerve coils. Cuticular sheaths (arrows) surround distal processes of sensory neurons. A) Trichogen cells, **tr**, secrete both the cuticle of the bristle shaft and of the new sheath about the distal processes, with multivesicular bodies, **mv**, and longitudinal fiber bundles, **f**. B) In the mature bristle shaft, **s**, the trichogen cell has receded. The sensory distal processes inside their cuticular sheath remain. C) Deep within the epidermis surrounded by an accessory cell, **ac**, a cuticular sheath provides a reservoir of sheath for a subsequent molt. D) Although the sheath may serve as protection, destruction * of neurotubules and loss of function may occur as ecdysis approaches (molting space, **m**). The calibration lines represent in A-D, 0.5 μ.

2A bristle morphogenesis by definition has not begun. Figures 2B-F are light micrographs of whole mounts of tergites showing bristles in various stages of morphogenesis.

The end of bristle morphogenesis in the individual has occurred when the new bristle has assumed its final form and starts thickening the shaft cuticle. In Figure 2D, bristle morphogenesis is nearing completion but shaft thickening has not begun. Figure 2E shows a morphogenetically complete new bristle, folded in the molting space of a tergite, with an obvious thickening of the shaft cuticle.

The release of epidermal muscle attachments to the old cuticle has occurred when the old cuticle is loose and can be detached from the new cuticle at the muscle attachments, Figure 2G. The assay for the event is to attempt removal of the mesonotal tergite; if the dorsal-ventral muscle attachments resist cuticle separation, the event has not passed. Figure 2F shows a longitudinal cross section of the hind edge of a tergite at a time close to the end of bristle morphogenesis as in Figure 2D; although a substantial molting space, is evident, the abdominal dorsal-ventral muscle attachments in this same animal have not yet released, Figure 2G.

Ecdysis is a process taking 20 minutes at 29° C climaxing the molting cycle. It begins with the swallowing of air to burst the old cuticle. This initial bursting of the cuticle was taken as the operational definition of the event ecdysis.

TABLE I

Estimation of the T_{50}'s of overlapping sets of quantal events and the standard deviation of departure from perfect synchrony. Event abbreviations are the same as in Figure 4.

Set	Event	Sample number	T_{50} ± standard error (hours)	Departure from perfect synchrony (hours)
A	pgfb	20	57.9 ± 3.5	7.6
	rcp	400	72.4 ± 0.6	12.0
B	rcp	371	68.5 ± 0.9	9.3
	e	120	124.3 ± 0.9	10.2
C	hcp	87	76.6 ± 1.8	10.8
	rcp	274	77.0 ± 0.9	8.7
	ptcp	91	87.4 ± 3.0	8.6
	e	35	136.1 ± 1.9	11.8
D	rcp	152	57.8 ± 2.8	10.6
	emit	100	75.0 ± 1.7	10.8
	e	47	125.2 ± 1.7	12.8
E	rcp	264	62.8 ± 0.9	9.8
	ptcp	270	68.8 ± 0.9	9.9
	nms	122	66.4 ± 1.9	9.6
	bmb	122	73.7 ± 1.0	7.4
	bme	122	86.3 ± 1.7	10.2
	e	169	121.8 ± 0.7	8.5
F	mar	400	104.2 ± 0.8	9.9
	e	50	130.0 ± 1.8	10.0

A map of quantal events

Since all events could not be assayed during any one experiment, overlapping sets of events were assayed and a summary comparison was computed statistically according to Finney (1952). Table I lists the six sets of events with their $T_{50's}$ and associated standard errors. The table also lists the standard deviation of the distribution of the event in time within the culture; this measures the departure of the event from perfect synchrony. The departure from perfect synchrony is a limiting factor in the usefulness of these cultures in studying developmental pheonmena. The average departure from perfect synchrony computed from Table I is about ten hours.

The overlapping sets of events listed in Table I are summarized diagrammatically in a temporal map, Figure 4. In this composite, events which could not be distinguished in time at the 5 per cent level of significance are grouped within a bracket. Thus the critical period for the head is indistinguishable in timing from the critical period for regeneration. Although statistical simultaneity does not mean absolute simultaneity, this close association of the two phenomena is suggestive of causal relationships. Another group of events which are closely associated with one another are the critical period for the prothorax, the end of epidermal mitoses and the appearance of nerve coils in the molting space. Since the prothoracic glands are a source of ecdysone, this close association also has possible causal significance. Besides pointing out groups of closely associated events, the mapping technique is useful in estimating the times when a particular event or process of interest is occurring with maximal frequency in a culture, for instance the map states that bristle morphogenesis takes approximately ten hours starting at 78 hours after feeding.

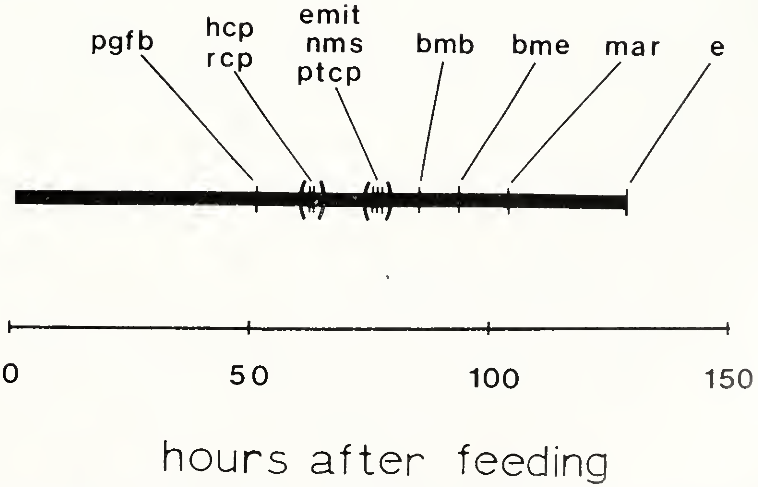


FIGURE 4. Temporal map of quantal events during the fourth instar of *Blattella germanica* raised at 30° C, showing: bmb, beginning of bristle morphogenesis; bme, end of bristle morphogenesis; e, ecdysis; emit, end of epidermal mitosis; hcp, head critical period; mar, muscle attachment release; nms, nerve coils in the molting space; pgfb, protein granules in the fat body; ptcp, prothoracic critical period; and rcp, regeneration critical period.

Epidermal cell proliferation

Since the beginning of proliferation of the epidermis could not be followed adequately as a quantal event it was decided to follow the increase of cell number quantitatively using the synchronous cultures. The intertergite region was used because of its lack of large numbers of organules. Groups of ten animals were fixed at various times after feeding, stained by the Feulgen reaction, and the density of nuclei per unit area of intertergite measured by *camera lucida*. Figure 5 shows the densities of generalized epidermal cell nuclei which were found at various times. During the first 44 hours after feeding, the nuclei per unit area declines approximately 10 per cent. This decrease in density could be accomplished by a combination of cell death, Figure 2E, and differentiation of epidermal cells into organules. Since few organules exist in the intertergite region the ten per cent decline has to be explained in terms of the observed cell death and perhaps differentiation into oenocytes.

According to Figure 5, between 48–84 hours after feeding the epidermis doubles its nuclear density. This apparent 36-hour proliferative phase in the intertergite region is most likely an overestimate. The overestimation is an artifact of following a rapid process in a less than perfectly synchronized (quasi-synchronized) culture of animals. The overestimate is indicated by some results obtained using colchicine in a non-parametric approach to estimating the length of the proliferative phase.

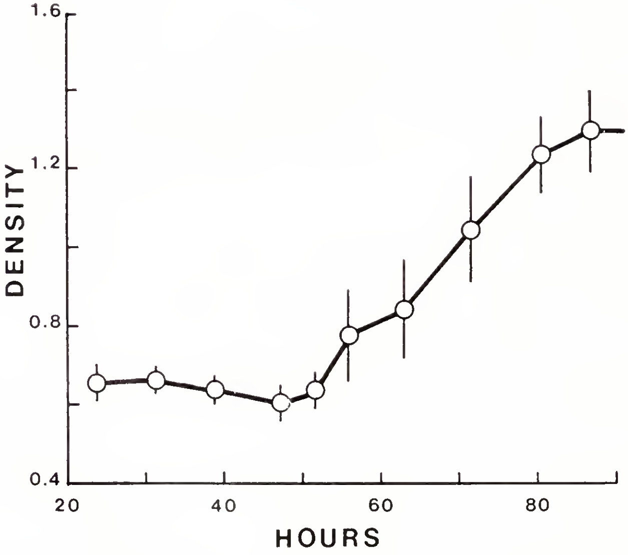


FIGURE 5. Density of generalized epidermal cell nuclei (nuclei/100 square microns) of the abdominal intertergite region during the fourth instar of *Blattella germanica*. Ecdysis occurred at a mean time of 130 hours after feeding. It was impossible to analyze samples after 86 hours of feeding due to shifting of the epidermis under the old cuticle. Since no colchicine mitoses are collected after this time, it is assumed that cell density levels off by 86 hours. Vertical bars represent one standard error on either side of the mean density for ten animals.

Using colchicine to collect mitoses for 2, 4 or 8 hours, a number of examples were obtained (2 animals out of 20 at the 8-hour collection) in which the eight hour collection spanned the peak of proliferation of the intertergite region. In these fortunate cases, Figure 1F, the majority of the generalized epidermal cells have undergone colchicine mitosis. Assuming that of the cells not in colchicine mitosis in Figure 1F, two-thirds had undergone mitosis prior to colchicine administration and one-third had yet to divide at fixation time, this would suggest that 65 per cent of the generalized epidermal cells divided during the eight-hour collection. If a linear increase of cell number is assumed this would allow all the cells to divide within about 12 hours. As will be shown subsequently, this is a reasonable estimate of the time for epidermal mitosis to be completed. The inconsistency of the 36-hour parametric estimate of proliferation time with the 12-hour non-parametric colchicine estimate points out a limitation of the use of these synchronous cultures for studying developmental processes.

Effect of regeneration on events of the molting cycle

The process of leg-autotomy has an all-or-none effect on the molting cycle. If the autotomy occurs before the critical period for regeneration, molting is delayed in order to regenerate the limb (O'Farrell and Stock, 1953). This delay of the molting cycle could be carried out in two different ways: either, the molting process could be slowed down by putting more time between events or the events of the molting phase could all be delayed jointly retaining their temporal spacing relative to each other. Experiments were performed to test these two alternative possibilities. If the molting cycle were simply slowed down by the regenerative process, then the observed delay of events early in the molting phase would be minimal while the delay in later events would be close to maximal. Six events were chosen to be studied: the appearance of protein granules in the fat body, critical period for regeneration, nerve coil appearance in the molting space, release of muscle attachments, ecdysis, and the midpoint of intertergite epidermal proliferation. The estimated delay of each of these events due to autotomy of a metathoracic leg is illustrated in Figure 6. All events studied associated with the molting phase were delayed jointly rather than being spaced farther apart. The appearance of protein granules in the fat body, Figure 6a, is not associated with the molting phase and shows no significant delay. Since intertergite epidermal proliferation, Figure 6c, was delayed by autotomy, it must be one of the first processes of what I have defined as the molting phase. Since the map time (Fig. 4) between the critical period for regeneration and the end of epidermal mitosis is approximately 17 hours, the colchicine estimate of 12 hours for epidermal proliferation of the intertergite is a reasonable one. Proliferative mitoses start earlier in the tergite region than in the intertergite and thus they probably begin about the same time as the critical periods for the head and regeneration.

DISCUSSION

The present paper attempts to establish an approach to using synchronous cockroach cultures in studying growth and development. In some systems growth and development are inextricably intertwined. In insects, growth and development

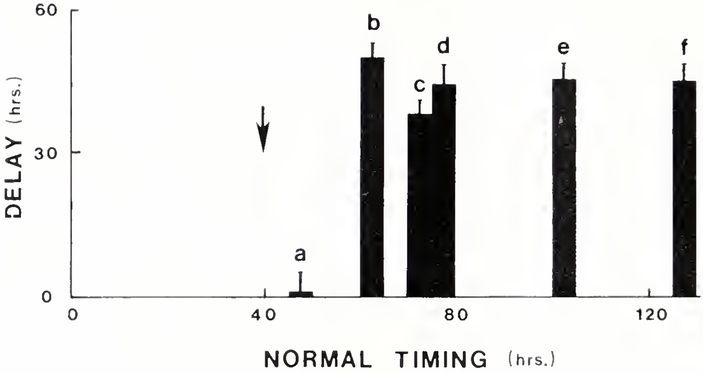


FIGURE 6. Delay of events due to leg regeneration. Delay was computed by subtracting the the normal timing of an event in controls from the delayed timing of the event in animals with a metathoracic leg autotomized at 40 hours after feeding (arrow). Events include: a) protein granules in the fat body; b) regeneration critical period; c) midpoint of intertergite cell proliferation; d) nerve coil appearance; e) muscle attachment release; and f) ecdysis. Vertical lines on each bar indicate the standard error of the delay.

are closely linked with molting physiology. It is demonstrated above that the differentiative and proliferative components of cockroach epidermal development occur in discrete phases. Differentiative processes such as the mitoses for new organules and cell death occur during the intermolt phase while proliferative mitoses occur during the early part of the molting phase (*cf.* Lawrence, 1966). By using an extrinsic control, leg autotomy, one can delay the events of the molting phase jointly and expand the time during which differentiation can occur. This separation and modulation of differentiative *vs.* proliferative phases provides a system for future testing and generalizing the concept of "quantal" *vs.* "proliferative" mitoses (Holtzer, Weintraub, Mayne and Mochan, 1972).

Although the differentiative divisions for bristles and dermal glands occur during the intermolt phase, the visible expression of their differentiated state awaits a morphogenetic period after proliferative mitoses have stopped. This beginning of the visible expression of the differentiated state, the epicuticular sculpturing of the new cuticle, is a molting phase phenomenon occurring after the prothoracic glands have made their last essential contribution to the initiation of molting.

The integrated growth and development of the cockroach involves both intermolt and molting phase phenomena but it is likely that intermolt phenomena control the direction that growth takes. Cockroaches approximately double in weight from instar to instar (Woodruff, 1938; Wigglesworth, 1972). This doubling of tissue mass of the animal requires only a $(2)^{2/3} = 1.49$ fold increase in surface epidermal cells. Despite the requirement for only a 1.49 increase, Figure 5 demonstrates an approximate doubling of the generalized epidermal cell population during the proliferative phase of epidermal cell division. This doubling is preceded by a decline in generalized epidermal cell number during the intermolt phase, attributable to both cell death and differentiation of epidermal cells into organules and oenocytes. The net result is a 1.49 increase in surface area from stage to stage.

It seems likely therefore that allometric and parametric growth of the cockroach exoskeleton can be controlled by the patterns of differentiative events and cell death that occur during the intermolt phase. This would allow a stereotyped response of the epidermal cells to the molting signals during the molting phase. This stereotyped response may include a proliferative division of every generalized epidermal cell.

The usefulness of synchronous cultures of cockroaches in studying the molting cycle has been demonstrated above as well as elsewhere (Kunkel and Lawler, 1974; Kunkel, 1975). The temporal map of events obtained by applying probit analysis to quantal data from synchronous cultures is useful in providing animals which are about to undergo a developmental process of particular interest. The degree of departure from absolute synchrony is a limiting factor in the types of approaches which can be used to study developmental phenomena. For example while attempting to study the relatively short term phenomenon, epidermal proliferation, it was shown that a misleading impression of the time for proliferation to be completed was obtained by plotting the mean epidermal cell density of random samples from the cultures against sampling time. A more accurate estimate of the twelve-hour proliferation time was obtained by a technique (colchicine injection) designed to measure the span of proliferation in individual animals. The synchronous culture technique was most valuable in this instance as a means of providing large numbers of animals undergoing epidermal proliferation. Obviously the greater the departure from perfect synchrony and the shorter the proliferative phase, the more animals one would have to inject to find at least one animal in which the colchicine collection of mitoses spanned the peak of proliferation. Despite these limitations in studying short term developmental processes, long term processes can be studied using the conventional approach of plotting means against sampling time (*cf.* Kunkel and Lawler, 1974).

The relationship of the critical periods for the head and prothorax to the events of the molting phase in the temporal map of cockroach development confirms similar phenomenology in other insects (Williams, 1948; Wigglesworth, 1952; Locke, 1970; Truman and Riddiford, 1974). The brain critical period presumably corresponds to the last time in the molting cycle at which a continued release of a brain hormone is necessary to assure eventual ecdysis. The brain has been shown to be responsible for the activation of the prothoracic glands in cockroaches (Gersh and Sturzebecker, 1970). Likewise the prothoracic critical period corresponds to the last time in the molting cycle that the prothoracic gland's product is secreted. Although these two phenomenological events serve as reference points in the molting cycle it is not at all certain what the concentrations of brain hormone and ecdysones are relative to these events, particularly since alternative sites of ecdysone metabolism have been suggested and documented (Locke, 1969; Weir, 1970; Nakanishi, Moriyama, Okauchi, Fujioka and Koreeda, 1972).

The close association of the regeneration critical period with the brain critical period suggests that the mechanism by which regeneration delays the molting cycle may be to delay the last brain function in initiating the molting cycle. This is contrary to prior suggestions that metabolism of ecdysone by the regenerating tissues was responsible for the delay (O'Farrell, Stock, Rae and Morgan, 1960; Pohley, 1964).

I am grateful to Professor Michael Locke for providing the stimulating environment in which this work was conceived and carried out. The work was supported by grants from the National Institutes of Health, GM 09960 to M. Locke and GM 33259 and AI 11269 to JGK.

SUMMARY

1. A temporal map of events during the molting cycle of *Blattella germanica* has been established.

2. The molting cycle can be divided into two discrete phases, the intermolt and the molting phase.

3. Molting phase events are delayed jointly when leg regeneration is induced.

4. Differentiative mitoses occur during the intermolt phase while proliferative mitoses occur at the beginning of the molting phase.

5. The coordinate growth and development of the epidermis seems to be controlled by a period of cell differentiation and death during the intermolt phase.

6. It is proposed that limb regeneration feeds back to delay molting by delaying brain hormone secretion.

LITERATURE CITED

CAVENEY, S., 1969. Muscle attachment related to cuticle architecture in Apterygota. *J. Cell Sci.*, **4**: 541–559.

DOANE, W. W., 1973. Role of hormones in insect development. Pages 291–497 in S. J. Counce and C. H. Waddington, Eds., *Developmental Systems: Insects, Vol. 2.* Academic Press, New York.

EDWARDS, J. S., 1966. Neural control of development in arthropods. Pages 95–110 in C. A. G. Wiersma, Ed., *Invertebrate Nervous Systems.* U. Chicago Press, Chicago.

EDWARDS, J. S., 1969. Postembryonic development and regeneration of the insect nervous system. *Advan. Insect Physiol.*, **6**: 97–137.

FINNEY, D. J., 1952. *Probit Analysis: a Statistical Treatment of the Sigmoid Response Curve.* [2nd ed.] Cambridge Univ. Press, London, 318 pp.

GERSH, M., AND J. STURZEBECHER, 1970. Experimentelle stimulierung der Zellularen aktivitat der prothakaldrusen von *Periplaneta americana* durch den aktivations faktor. *J. Insect Physiol.*, **16**: 1813–1826.

HOLTZER, H., H. WEINTRAUB, R. MAYNE, AND B. MOCHAN, 1972. The cell cycle, cell lineage and cell differentiation. *Curr. Top. Dev. Biol.*, **7**: 229–256.

JENKIN, P. M., AND H. E. HINTON, 1966. Apolysis in arthropod moulting cycles. *Nature,* **211**: 871.

KUNKEL, J. G., 1966. Development and availability of food in the German Cockroach, *Blattella germanica.* (L.) *J. Insect Physiol.*, **12**: 227–235.

KUNKEL, J. G., 1973. Gonadotrophic effect of juvenile hormone in *Blatella germanica:* A rapid, simple quantitative bioassay. *J. Insect Physiol.*, **19**: 1285–1297.

KUNKEL, J. G., 1975. Larval-specific protein in the order Dictyoptera - II. Antagonistic effects of ecdysone and regeneration on LSP concentration in the hemolymph of the oriental cockroach, *Blatta orientalis. Comp. Biochem. Physiol.*, in press.

KUNKEL, J. G., AND D. M. LAWLER, 1974. Larval-specific serum protein in the order Dictyoptera - I. Immunological characterization in larval *Blattella germanica* and cross-reaction throughout the order. *Comp. Biochem. Physiol.*, **47B**: 697–710.

LAI-FOOK, J., 1967. The structure of developing muscle insertions in an insect. *J. Morphol,* **123**: 503–528.

LAWRENCE, P. A., 1966. Development and determination of hairs and bristles in the Milkweed Bug, *Oncopeltus fasciatus* (Lygaeidae, Hemiptera). *J. Cell Sci.,* **1**: 475–498.

LAWRENCE, P. A., 1973. The development of spatial patterns in integument of insects. Pages 157–209 in S. J. Counce and C. H. Waddington, Eds., *Developmental Systems: Insects, Vol. 2.* Academic Press, New York.

LOCKE, M., 1966. The structure and formation of the cuticulin layer in the epicuticle of an insect, *Calpodes ethlius* (Lepidoptera, Hesperiidae). *J. Morphol.,* **118**: 461–494.

LOCKE, M., 1967. The development of patterns in the integument of insects. *Advan. Morphogenesis,* **6**: 33–88.

LOCKE, M., 1969. The ultrastructure of the oenocytes in the molt/intermolt cycle of an insect. *Tissue Cell,* **1**: 103–154.

LOCKE, M., 1970. The molt/intermolt cycle in the epidermis and other tissues of an insect *Calpodes ethlius* (Leidoptera, Hesperiidae). *Tissue Cell,* **2**: 197–223.

NAKANISHI, K., H. MORIYAMA, T. OKAUCHI, S. FUJIOKA, AND M. KOREEDA, 1972. Biosynthesis of α and β ecdysones from cholesterol outside the prothoracic gland in *Bombyx mori. Science,* **176**: 51–52.

O'FARRELL, A. F., AND A. STOCK, 1953. Regeneration and the molting cycle in *Blattella germanica* (L.) I. single regeneration in the first instar. *Austr. J. Biol. Sci.,* **6**: 485–500.

O'FARRELL, A. F., A. STOCK, C. A. RAE, AND S. A. MORGAN, 1960. Regeneration and development in the cockroach *B. germanica. Acta Soc. Entomol. Czech.,* **57**: 317–324.

POHLEY, H-J., 1964. Regeneration and the molting cycle in *Ephestia kuhniella.* Pages 324–330 in V. Kiortsis and H. A. L. Trampusch, Eds., *Regeneration in Animals.* North Holland Pub. Co., Amsterdam.

ROTH, L. M., AND W. H. STAHL, 1956. Tergal and cercal secretion of *Blatta orientalis. Science,* **123**: 798–799.

SLIFER, E. H., 1970. The structure of arthropod chemoreceptors. *Annu. Rev. Entomol.,* **15**: 121–142.

TRUMAN, J. W., AND L. M. RIDDIFORD, 1974. Physiology of insect rhythms III. The temporal organization of the endocrine events underlying pupation of the tobacco horn worm. *J. Exp. Biol.,* **60**: 371–382.

WEIR, S. B., 1970. Control of moulting in an insect. *Nature,* **228**: 580–581.

WIGGLESWORTH, V. B., 1934. The physiology of ecdysis in *Rhodnius prolixus* Hemiptera II. Factors controlling molting and metamorphosis. *Quart. J. Microscop. Sci.,* **79**: 91–121.

WIGGLESWORTH, V. B., 1942. The significance of "chromatic droplets" in the growth of insects. *Quart. J. Microscop. Sci.,* **83**: 141–152.

WIGGLESWORTH, V. B., 1952. The thoracic gland in *Rhodnius prolixus* and its role in moulting. *J. Exp. Biol.,* **29**: 561–570.

WIGGLESWORTH, V. B., 1953. The origin of sensory neurons in an insect, *Rhodnius prolixus* (Hempitera). *Quart. J. Microscop. Sci.,* **94**: 93–112.

WIGGLESWORTH, V. B., 1957. The use of osmium in the fixation and staining of tissues. *Proc. R. Soc. London, Series B,* **147**: 185–199.

WIGGLESWORTH, V. B., 1961. The epidermal cell. In J. A. Ramsay and V. B. Wigglesworth, Eds., *The Cell and the Organism.* Cambridge University Press, Cambridge.

WIGGLESWORTH, V. B., 1972. *The Principles of Insect Physiology.* [7th ed.], Chapman and Hall, London, 827 pp.

WILLIAMS, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia. Biol. Bull.,* **90**: 234–243.

WILLIAMS, C. M., 1948. Extrinsic control of morphogenesis as illustrated in the metamorphosis of insects. *Growth Symp.,* **8**: 61–74.

WILLIS, J. H., 1974. Morphogenetic action of insect hormones. *Annu. Rev. Entomol.,* **19**: 97–115.

WOODRUFF, L. C., 1938. Normal growth rate of *Blattella. J. Exp. Zool.,* **79**: 145–165.

WYATT, G. R., 1972. Insect Hormones. Pages 385–490 in G. Litwack, Ed., *Biochemical Actions of Hormones Vol. II.* Academic Press, New York.

Reference: *Biol. Bull.*, **148**: 274–285. (April, 1975)

SPECIFIC DEATH SITES IN A *DROSOPHILA* POPULATION CAGE

ROGER MILKMAN

Department of Zoology, The University of Iowa, Iowa City, Iowa 52242

I wish to report the observation that when population cages of *Drosophila melanogaster* are provided with empty vials, more than half of the flies die in these vials rather than elsewhere. This phenomenon has been followed continuously over a period of some 1½ years, in a dozen cages, and under a variety of conditions. It bears on the nature of territoriality and on the control of population density.

MATERIALS AND METHODS

Populations of *Drosophila melanogaster* were established in lucite population cages (boxes 135 × 110 × 450 mm o.d., on 115 mm supports, screen-vented at each end, and fitted with 20 standard 25 × 95 mm glass culture vials in two rows). The cages were located on a counter in my laboratory at room temperature. Light was not controlled. All but two culture vials contained about 10 cc of standard culture medium and were replaced at staggered intervals, so that a complete age array of *Drosophila cultures* was present at all times. The remaining two vials were empty; these were placed in terminal positions and removed at certain intervals for counting. Before the empty "death" vials were removed for counting, they were tapped repeatedly to encourage flies to depart that might be casual transients. The remaining flies were etherized; those that recovered were designated "moribund," and those that did not were called "dead." Ideally, the ether would finish off no living flies, and this appears to have been essentially the case: most flies in the death vials were clearly dead. The moribund flies exhibited characteristic behavior.

RESULTS

A log of the running observations over a 16-month period is presented in Figure 1. The data were collected, first from the death vials of 9, then from 4 cages, and they consist of two variables: number of dead flies per vial and number of moribund flies per vial at the time of counting. These data do not include flies dying or becoming moribund elsewhere in the cages.

Table I presents these and derivative data for certain time periods. At the outset, Standard Period 1, the two death vials in each cage were at the end facing into the room. In Figure 1, a sharp oscillation can be seen: the right hand vials were nearer the major source of light, a window, and they almost invariably contained more flies.

The cages were then reversed, so that the death vials were now against the wall, where it was darker. Now the number of flies dying in the death vials dropped sharply, although the number of moribund flies did not. The right-left difference disappeared, also. When the cages were turned around once more

TABLE I

Calculation of death rates

Sample period	Experimental time (days)	Dead (N)	Dead day/vial	Moribund (N)	Dead + Moribund	Vial days	Replicates (N)	Death rate**
Standard I	64–153	45,397	26.97	15,366	60,763	187	9	36.10
Reverse*	164–216	3,842	8.50	3,320	7,162	113	4	15.85
Standard II	223–263	8,872	25.49	3,149	12,021	87	4	34.54
Foil and Hood*	271–331	12,460	24.34	5,088	17,548	128	4	34.27

* See text and Figure 1.

** Dead and Moribund Flies divided by (Vial Days × Replicates).

(Standard Period II), the results returned to their original values, including the right-left difference (Figure 1). Next, the death vials were covered with foil (D) and then additionally the right hand sides of the cages were covered with foil (E). Finally (F), foil or mylar hoods were placed over each cage. Now the observations were similar to those seen under the initial conditions, except that the oscillations due to right-left differences were not seen. To interpret these results simply, it appears that light does attract the flies, even the moribund ones, but the influence of light is an epiphenomenon: in the near absence of light, the dying flies accumulate in the death vials to the same extent as in light.

It may be noted that the death vial floors occupy about 1.7% of the total floor area of the cage, so that the accumulation of moribund and dead flies in these vials is anything but random. Vials placed horizontally in a port at the end of the cages accumulated very few dead flies (1.6 per vial per day).

Table II illustrates the ratio between moribund flies per vial (at time of sampling) and dead and moribund flies per vial per day. In a steady state situation, the rate of arrival of moribund flies equals the death rate. The ratio of moribund flies present at any time to the death rate should equal the duration of the moribund period, and this works out to about 46 hours. In contrast, in a recent experiment, healthy flies lived a median time of 23 hours in empty vials under ambient conditions of temperature and humidity, and 34 hours if the cotton plug was moistened. Since the moribund flies entering the death vials are not even representative healthy flies, we might have expected them to last less than 24 hours. The fact that they appear to live on for almost 2 days suggests that some moribund flies do reenter the arena and eat once or occasionally more often.

The distinction between moribund flies and ordinary healthy flies is illustrated by the results of the following experiment. Over 400 moribund flies (see materials and methods for criterion) were collected and placed in ordinary food vials, at the low density of 20 per vial. The survivors in each vial were transferred to a new vial at roughly 5-day intervals. Median survival time was 26 days, and the survival curve was smooth, not stepped. Thus, the flies seem to have constituted a fairly continuously variable group, not a mixture of more than one distinct class. A comparable group of over 400 flies attracted from the main part of the cage by light and treated similarly showed a median survival time of 52 days. Again the curve was smooth and not stepped. Moreover, abundant

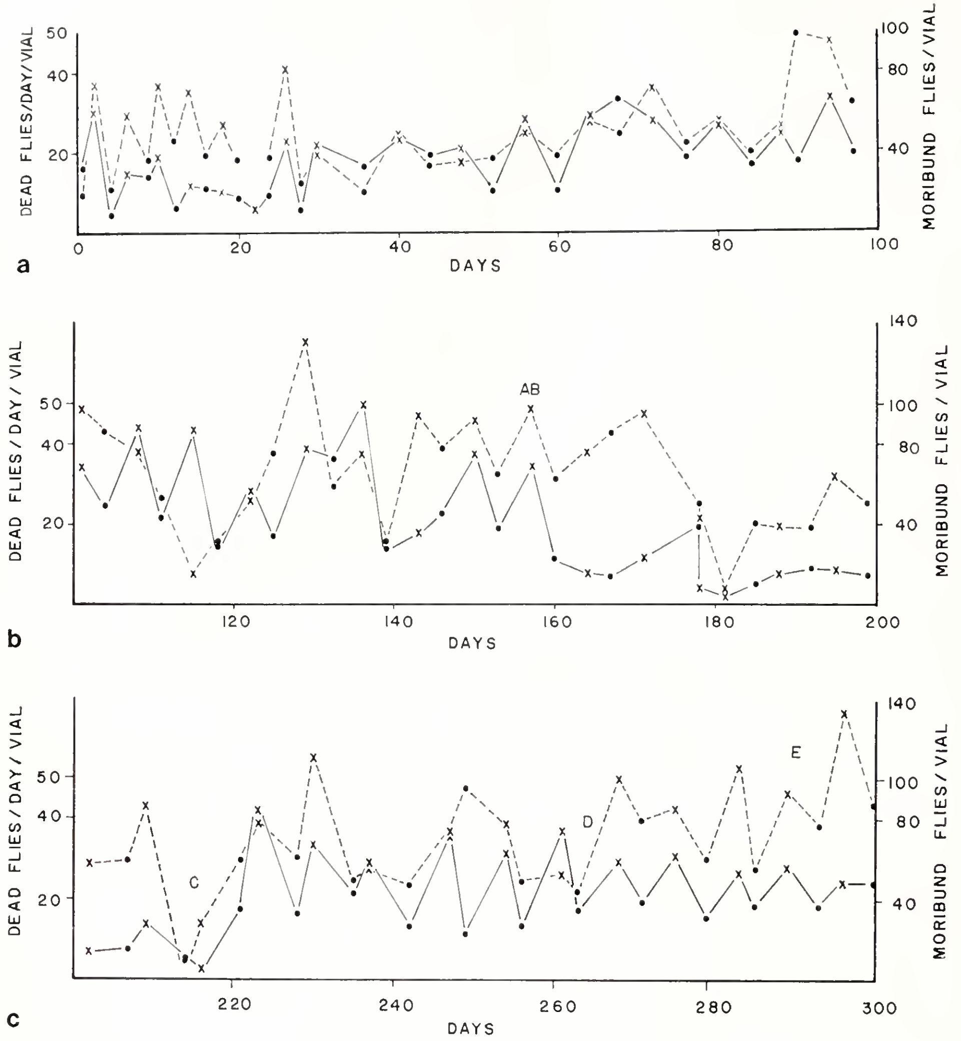
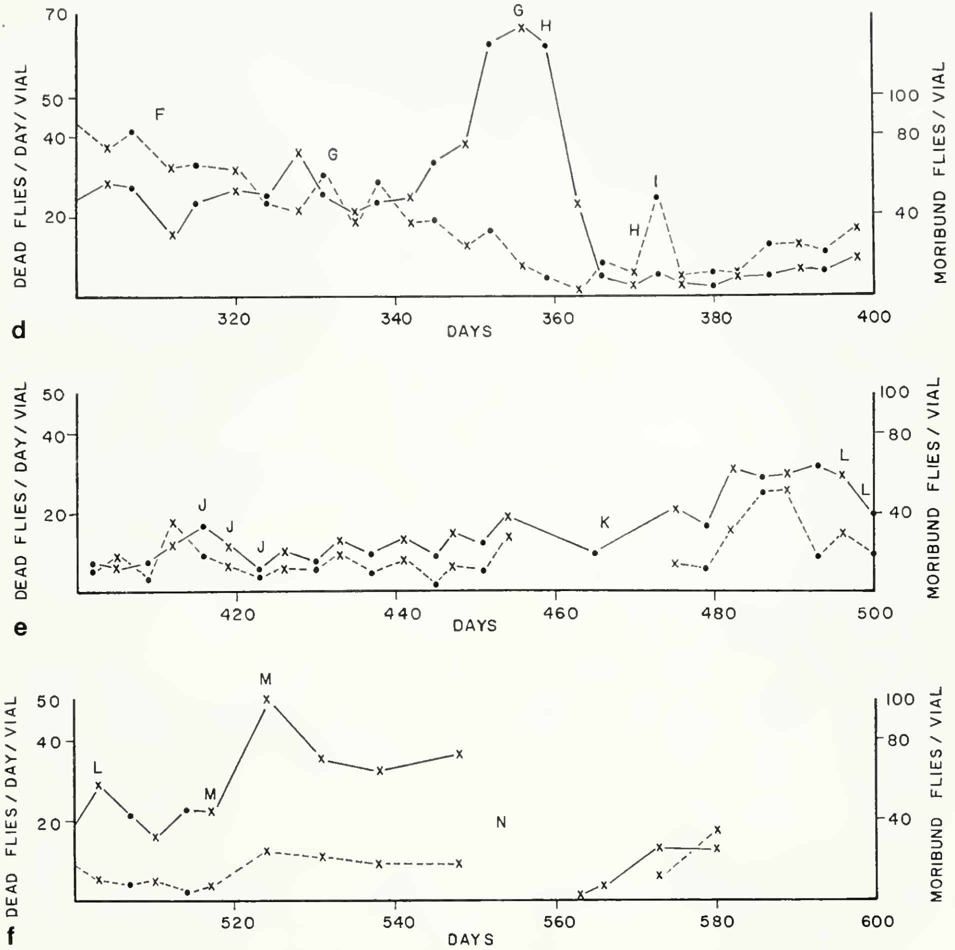


FIGURE 1. Numbers of dead flies per vial per day (continuous line) and number of flies per vial (dashed line) in right (cross) and left (filled circle) death vials. For Day 0–156, samples were averaged from 9 cages. At A (Day 157), samples were averaged from 4 cages from this date on. At B (Day 157), cages were turned around, so that death vials were nearest the wall. At C (Day 216), cages were turned back to original positions. At D (Day 264), death vials were covered with aluminum foil. At E (Day 290), the right side of each cage (side nearer the light) was covered with aluminum foil. At F (Day 310), the entire cage and food vials were shielded by loose fitting foil or mylar hood. Through G (Days 331 through 356), no new food vials were added in this period; no culture vials were removed. At each H (Days 359, 363, 366, 370), 4 fresh food vials were added to each cage on each of these days. At I (Day 373), all aluminum foil coverings were removed from cages. On J (Days 416, 419), short (45 mm deep) death vials replaced the regular vials removed for counting; the first counts from short vials were made on day 423. At K



(Day 466), all short death vials were replaced by regular (95 mm deep) vials. On L (Days 496, 500), short death vials replaced the regular death vials removed for counting; the first counts from short vials were made on day 503. At M (Day 517), both short death vials were removed. A single regular death vial was placed in each cage; the first counts from the single vials were made on day 524. At N (Day 553), all vials from each cage were cleared of flies; each set was placed in a clean, empty cage. Between days 380 and 400 substantial samples were taken from the cages for purposes of genotyping. The low values subsequently seen can be attributed to the reduced population size (in addition to the addition of a large number of fresh food vials).

progeny were left by the transferred moribund flies, and subsequent specific tests of fertility showed that essentially all moribund flies of both sexes were fertile when transferred to uncrowded conditions.

The absence of any indication of sharp heterogeneity among the moribund flies is important. While a two-step death curve would have indicated a mixture

TABLE II

Estimates of length of moribund period

Sample period	Moribund Flies per vial	Death Rate per vial	M/d**	
			Days	Hours
Standard I	65.67	36.10	1.82	44
Reverse	51.88	15.85	3.27	78
Standard II	65.60	34.54	1.90	46
Foil and Hood	74.82	34.27	2.18	52
Average, Rows 1, 3, and 4	67.44	35.52*	1.90	46

* Or 71.04 per cage.

** Number of moribund flies divided by death rate.

of dying and normal (presumably transient) flies, or else a two-step degenerative process, the apparent homogeneity of the moribund flies permits them to be treated tentatively as a single class. Thus the apparent 2-day duration of the moribund period must be taken as representative of the experience of each fly. Accordingly, the moribund flies, by leaving the death vials on occasion, may be capable of responding to altered conditions in the cage. Perhaps they can remain in the cage proper if its density has dropped, or if the competition has lightened.

A test of this possibility was performed by exchanging the death vials of a wild-type cage and one containing a population of *vestigial sepia* (*vg se*) flies. The latter strain is unable to compete with wild flies except under unusual circumstances. When the *vg se* death vials were placed in the wild cage, no flies left them to enter the cage. It should be noted that flies leave vials primarily on foot, so the absence of functional wings is not in itself a big drawback to the *vg se* flies. In the reciprocal maneuver, nothing happened at once, but eventually some moribund wild flies did leave their death vials and enter the *vg se* cage proper. Their movements were slow and halting. Eventually a few reached food. Some time after, and looking more robust (moving faster and appearing less shriveled), they seemed to take up positions and to threaten approaching *vg se* flies. These observations are anecdotal and must be understood in this spirit; for what they are worth, the wild flies executed sharp wing movements at the approach of *vg se* flies, and the latter turned away. Furthermore, a large number of *vg se* flies were soon in the death vials. Systematic observations of such encounters obviously constitute an important next step in the investigation of this whole subject. Eighteen days after the transfer, large numbers of wild flies began to appear in the cage, obviously progeny of the moribund wild flies that had left the death vials.

It should be noted that the criterion of moribundity depends in part on the intensity and persistence with which death vials are tapped and rattled before being removed. While light tapping results in the prompt departure of some flies, continued and heavier rattling causes more flies to leave. After a recent decision to increase the severity of the rattling, the numbers and proportions of flies counted as moribund have dropped. Estimates of durations of the moribund period, each averaged over several weeks' observations, are given in Table III.

TABLE III

Estimates of length of moribund period, considering only flies remaining after prolonged and intensive rattling

Cages	Conditions	M/d* (hours)
1,3,5,7	Standard	24.8
1,3,5,7	Vials in mid-cage position	42.5
2,4,6,8	Standard	23.7
2,4,6,8	Short vials	8.0
2,4,6,8	One regular vial per cage	12.2

* Number of moribund flies divided by death rate.

Another experiment employed genetically marked flies to get an idea of the age-distribution of the dying flies. In each of two cages, six vials of *net black cinnabar brown* (*n b cn bw*) flies were inserted for a period of three days, replacing regular culture vials. These *n b cn bw* cultures were just ready to produce their first adults; the strain is quite robust and competes well with wild flies in the laboratory. Eventually, a total of 167 *n b cn bw* flies appeared in the death vials, and of these 64 were found in the first three days. The rest appeared sporadically in declining numbers until 40 days later, after which none appeared for a considerable period. Meanwhile 72 had been found in the cage food vials that were replaced on a regular schedule. Since the *n b cn bw* cultures contained no adults when placed in the cage, 64 of the 239 that hatched during the three-day period entered the death vials at an early age: three days at most, and many must have been much younger. A few looked quite callow when found. Thus, the death vials receive adult flies of all ages, and if it is the weaker flies that become moribund, age is not the only cause of the weakness. Both these cages, a year later, contained numerous flies showing one or more of the recessive mutant phenotypes. (Other cages have been maintained with polymorphisms at these loci—and fairly even allele frequencies—for three years in our laboratory). Thus the appearance of large numbers of young *n b cn bw* flies in the death vials probably reflects the events characteristic of normal populations rather than the quick demise of an inferior stock.

In order to estimate the rate of emergence of adult flies (and therefore the death rate, given a steady state), half of the producing culture vials were removed from each of two cages. Emergent flies were counted and removed daily for three days, leading to an estimate of emergence per day of 86 for one cage and 198 for the other. The flies in each cage were counted directly *in situ* (this is tedious but practical). The first cage contained about 1570 flies; the other, 4010. Mean adult longevity thus is computed to be 1570/86 = 18 days for the first cage and 4010/198 = 20 days for the second. The remaining 9 cages, similarly counted, averaged 2468 flies (range = 1850–3355). With a mean adult longevity of 19 days, a mean daily emergence (and death) rate of 130 is indicated. Over long periods of time (Table II), the death rate for the two death vials of a cage, taken together, averages over 71. In computing the death rate, the moribund flies must of course be included. The total number of dead and moribund flies is divided by the duration of the vial's stay in the cage and by the number of replicate vials. The mean death rate of 71 flies per cage per day includes all periods except the "reversed" period.

When cages contain no empty vials, the newest food vials often contain very large numbers of males and females. Many of these are dead and dying, so that in ordinary cages each food vial serves as a graveyard (as well as a nursery simultaneously) in its turn. This dual role is illustrated by the following observation. When one rattles a death vial repeatedly, some flies leave, but many remain. These are of course the moribund flies. When one rattles a crowded newly-placed food vial in the same cage, essentially all the flies leave. The critical test is now made on newly-placed food vials in cages without death vials: many flies remain in the vial. As in the death vials, they may climb half way up the vial, but they keep dropping back. These observations were made 3–20 hours after emplacement of the vials. Clearly, the immediate distinction resides in the flies and not in the physical differences between empty vials and those containing food. In this context it may also be noted that short vials (45 mm deep) seem to serve equally well as death vials. Moreover, recent experiments show that a *single* death vial accumulates over half the flies that die in the cage. Regular-sized (25 × 95 mm) vials were used in 4 cages, whose adult emergence rate was later measured by removal of all producing vials and the counting of all emergent flies each day for two days; then all the vials from each cage were placed in a clean cage, whose population was then counted directly after several days. After the vials had been removed from the old cages, the flies in each cage were removed, sexed, and counted. From these counts, and from adult emergence rates, adult longevities were estimated for each sex, as shown in Table IV. These estimates are considerably higher than those made over a year earlier when the cage populations were newer. The average death vial rate was about 41 at this time.

TABLE IV

Mean adult longevity estimates for four $1\frac{1}{2}$-year-old cage populations

Sex	Mean adult emergence per cage per day	Mean cage population	Mean adult longevity, days
F	37.4*	1133.0**	30.3
M	33.5	1419.5**	42.4

* Proportion of females, 0.527; proportion in three other cages, 0.536.

** Some flies of unknown sex were added in the proportions observed; they constituted well below 5% of the entire sample.

While the numbers are dramatically lower in the new food vials of cages containing empty vials, it is likely that some flies do die preferentially here as well. Furthermore, under conditions where the medium pulls away from the glass, many flies are trapped in the resultant crevices. The early estimates of death rates (130 overall and 71 in the death vials) differ by about 60. Over a year later the respective estimates of 71 and 41 are lower but in a comparable ratio. Those flies that do not die in the death vials presumably find similar, though ostensibly less specific, conditions, such as the new food vials may offer. And others may die in random places. The proportions of these respective groups are not known.

When death vials are removed for counting, they are plugged with cotton and left for up to an hour before the counts are made. At the end of this time, some of the living flies look anything but moribund: they are active and well coordinated in every obvious respect. Indeed, some flies begin to look normal a few minutes after removal of the death vial. This observation indicates the possibility of a depressing olfactory—or even auditory—stimulus from the cage, a stimulus whose removal is accompanied by a recovery of vitality by some of the moribund flies.

We have tried without success to analyze emigration in sets of vials connected by tubes of various sizes. A variety of mixed populations were used. From these experiments and from the observation of cages, where most of the healthy flies are not in the culture vials, we have concluded that an element is missing from such theaters, and that is abundant flat surface. Accordingly, our further experiments are being done in standard and special cages. In the course of these experiments, a distinct additional form of behavior has been noted and studied. It will be described in a subsequent publication.

Discussion

Clearly, the flies accumulate in a specific place in the cages before dying. What do we know about the process, and what questions remain?

First of all, the death vials are not merely traps. Some flies can easily leave the vials, and healthy flies placed in empty vials have no trouble escaping. As noted, shorter vials serve well as death vials. Also, if we take the estimates of the duration of the moribund period seriously, moribund flies do leave the death vials at least once or twice on the average to get food and water. To recapitulate the reasoning, moribund flies placed in closed food vials survive well and behave as a unimodal class; they live only half as long as ordinary healthy flies under these conditions. Healthy flies live only about a day without food, the moribund flies appear to live about two days in the death vials. Thus they must be going out for sustenance once in a while. The decisive observation has not been made, however: moribund flies must be seen to leave the death vials, enter food vials, and return. Obviously, too, there must be a progressive decline of vitality toward death. Presumably, the flies that do not leave the death vials even after extreme disturbance are in a weaker state than those that do. Nevertheless, the state of moribundity holds interest in its initial as well as more extreme stages.

The behavior of the moribund flies suggests impending death. Their movements are erratic, uncoordinated, often non-adaptive, and quite reminiscent of the familiar behavior of moribund houseflies. But what organized activity brings them to this particular spot? Where have the flies been just before entering the death vials? What interactions, if any, with other flies preceded their departure? Is the departure a form of emigration, a form of suicide, a set of both, or neither?

It seems to me that the flies are emigrating. The unusual circumstances make them emigrate into captivity, but that may well be a frequent occurrence in nature, too. The time comes when each fly has to take his chances elsewhere. And perhaps this time is announced to the fly in a series of interactions whose model is seen in territorial behavior.

Naturally these observations will call to mind what Wynne-Edwards (1962) has said so well. Perhaps the continuous nature of the emigration is novel, however. Wynne-Edwards emphasized the "safety-valve" aspect of emigration and discussed it solely as an intermittent (regular or irregular, but not continuous) activity in populations. In other discussions (Dingle, 1972; Johnson, 1969; Krebs, Gaines, Keller, Myers, and Tamarin, 1973) as well, the sporadic or cyclical aspects of emigration are emphasized. The *Drosophila* cages, in contrast, present an example of continuous, though modulated, emigration. When the end-point of migration is an empty vial, socially-induced emigration and socially-induced mortality are one and the same thing.

There is nothing new about the departure of queen bees from a hive; the exclusion of young gulls or young male blackbirds from specific territories; or the flight of refugees before human conquerors. In general, however, we think of territories as more or less fixed. The stickleback knows his borders; so do many birds. Nevertheless, there is nothing essential to the fixity of territory. Granted, permanent territories add stability to a colony, and they are easy to observe. But a given area can be subdivided among a number of moving individuals, each of whom may during a stationary period or even while in motion drive away others who come too close. If this does not fit the conventional concept of territoriality, then it is certainly prototerritoriality. In any event, I believe such behavior may underlie the accumulation of the flies in the death vials.

When a fly approaches a stationary male, the latter flutters its wings, and the former generally moves on. Often one male will pursue another, poking at its abdomen as in courtship. These interactions are the type that could add up to a departure to the death vial, but of course we have no explicit information on the question. But whatever the specific interactions, the model of an area dynamically saturated with floating territories (or "personal space") may serve as a guide to the design of further experiments. While such a model does not answer a lot of specific questions (*e.g.*, how does a fly know when to go to the death vial?), it makes them quite analogous to questions for which we have straightforward answers (a herring gull flees from a hostile opponent whose threat behavior indicates superior strength and a determination to attack). The critical observation here is the path of a fly to the death vial. No doubt a movie played backwards could identify the beginning.

Aggressive behavior and non-random spacing have been noted in experimental populations of *Drosophila* from time to time. While Hay (1973) did not discriminate among a variety of activities in *D. melanogaster*, he was aware that some of them were likely to fend off other flies. Manning's (1959) description of wing-flicking fits what I have referred to earlier; Connolly (1968) recognized uniform spacing as possibly significant and attributed an increase in "preening" (activity without translocation) to the visual perception of other flies. Sexton and Stalker (1961) noted that the upper surface of a *cylindrical* chamber was choice.

In their experiments, at most 183 flies occupied the ceiling, even with as many as 1,000 flies in the chamber. Under crowded conditions, they report intriguingly, some flies were on the wall and many were piled up on the floor. The extension of legs seemed to ward off other flies; this behavior was apparent when another fly came within about 5 mm of a stationary individual. While no specific (permanent) territory was defended, Sexton and Stalker (1961) noted that the uniform spacing might nevertheless "reduce competition." Naylor (1959) showed clearly that *Tribolium confusum* (flour beetle) populations have a density-dependent spacing pattern, with crowding resulting in spacing more uniform than a random distribution. Clark and Evans (1954) treat the analysis of spacing in a clear and thorough way. These several observations and interpretations of spacing patterns can usefully be thought of together with the phenomenon of emigration, which Lidicker (1962), among others, has considered as a possible component of a mechanism regulating population density. The experiments of Sakai, Narise, Haraizumi, and Iyama (1958) on emigration in *Drosophila* deal with unit time periods considerably longer than those employed here; more important, perhaps, discrete culture generations were employed, and they used large vials connected by tubes. Taken together, the observations cited indicate the common use of behavior resulting in even spacing, the possible coupling of spacing to emigration, and the occurrence of emigration on a scale sufficient to have a major impact on population density.

The importance of this behavior lies in its role in the control of population density. Mass-action-limits debilitate the population, and in the absence of grazing and similar causes of death, mechanisms must be found to prevent all the individuals from living on the brink of starvation. In fact, cage flies are invariably a lot larger than the tiny but fertile individuals one can produce experimentally by a judicious manipulation of the medium. As Wynne-Edwards (1962, p. 10) says, ". . . the ceiling is normally imposed, and the level indefinitely maintained, while the members of the population are in good health—sometimes actually fat—and leading normal lives." There are two evident behavior patterns in *Drosophila* cages that make for a less-than-maximal partitioning of the environment. First, many larvae leave the food prematurely under crowded conditions. Circumstances arise which cause them to take their chances elsewhere; in a cage, they starve on the sides of the vials. This leaves enough food (and perhaps other resources) for those who remain to metamorphose into robust adults. And secondly, adult flies go to the death vials before it is physiologically necessary to die. In each case, the individuals die on cue. Cues, weak signals amplified by the receiver, are essential to regulation.

Why should an individual face certain death rather than settle for a meager existence? The answer, again, lies in territoriality. Even where death by attack is not possible, evolution can program individuals to prefer a step towards death to receiving a certain stimulus, to choose isolation over insult, to find challenge more agonizing than deprivation. The context of this preference may lie in the same appreciation of superior competition that turns many a bird, insect, and mammal away from a displaying rival. Then what of the *insensitive* inferior individual? He may survive longer, but he will not prosper. The cost to the population will not be great, and there is nothing to make the property of insensitivity spread. Also, it may be that no single behavioral mutation can be so deft as to leave an individual immune to the signal to go away, yet perfectly capable of using all other information from his environment.

Most individual organisms do not live a continuously marginal existence. Resources are usually partitioned so that each share is well above the minimal subsistence level. While many natural populations may be kept below deleterious densities by outside forces, and while others may oscillate and occasionally be cut back by actually reaching such densities, a *Drosophila* cage population's density is limited by factors intrinsic to the population itself. Individual larvae and individual adults must be able to choose a new quasi-developmental track (towards moribundity) in response to environmental cues, in the same sense that crowded locusts choose to emigrate, certain termites choose to become soldiers, and tissues in a broken salmander limb choose to regenerate. At a fork in the road, one direction is taken; the other is not. This is not rational choice, but it is a choice.

Presumably a cage can be made to have a density sufficiently low to eliminate death on cue. At such a density, perhaps the death vials would receive only their random share of moribund flies. Alternatively, perhaps *all* moribund flies are inclined to seek out such death sites, whether or not they die before the final possible time. In any event, the nature of the cues and of the flies' initial responses to them should be of great intrinsic and general significance.

In various sorts of population cage studies, the ability to monitor death will have useful applications. For example, a comparison of genotype frequencies between newly-emerged and newly-dead flies will indicate whether the population is in a steady state. If it is, comparison of genotype frequencies between newly-dead flies and the general population can indicate whether certain genotypes influence longevity and therefore, presumably, fitness. Indeed, longevity may be a critical variable in the operation of selection—and perhaps the maintenance of genic polymorphism—in cage- and natural-populations.

Of course the death-vial death rate is apparently just over half the overall death rate, and it may well be a variable fraction, at that; but if conditions can be devised which increase and stabilize this fraction, then the resultant ease of obtaining death-rate estimates will be quite valuable. It is obviously far easier and less disruptive to monitor death vials than to monitor producing culture vials. Since one or the other must be done to estimate generation time (and in turn track progress toward linkage equilibrium, for example), the development of conditions favoring the death vials as the place of death is most desirable.

The assistance of Judith Barnes, Jessica Ingstad, Lonnie Kennedy, Mary Kratoska, and Beverly Pennell is gratefully acknowledged. This work was supported in part by NIH Grant GM-18967.

SUMMARY

More than half the flies that die in a *Drosophila melanogaster* population cage do so in empty vials if they are provided. Before dying, the flies exhibit characteristic erratic behavior; if placed in uncrowded conditions they are fertile and

they live for several weeks. This phenomenon is neither light-dependent nor exclusively age-dependent. Crowding is clearly important.

It appears that the healthier flies maintain moving territories, keeping others at a distance and thus minimizing crowding. The others emigrate, in this case into a resourceless chamber, so that socially-induced emigration becomes socially-induced death. This physiologically unnecessary death is viewed as a component of an intrinsic population-density-regulating mechanism in *Drosophila melanogaster,* and presumably in many other organisms that have no fixed territories.

LITERATURE CITED

CLARK, P. J., AND F. C. EVANS, 1954. Distance to nearest neighbor as a measure of spatial relationships in a population. *Ecology,* **35**: 445–453.

CONNOLLY, K. J., 1968. The social facilitation of preening behaviour in *Drosophila melanogaster. Animal Behaviour,* **16**: 385–391.

DINGLE, H., 1972. Migration strategies of insects. *Science,* **175**: 1327–1335.

HAY, D. A., 1973. Effects of genetic variation and culture conditions on the social behavior of *Drosophila melanogaster. Behavior Genetics,* **3**: 107–119.

JOHNSON, G. C., 1969. *Migration and dispersal of insects by flight.* Methuen, London, 763 pp.

KREBS, C. J., M. S. GAINES, B. L. KELLER, J. H. MYERS, AND R. H. TAMARIN, 1973. Population cycles in small rodents. *Science,* **179**: 35–41.

LIDICKER, W. Z., JR., 1962. Emigration as a possible mechanism permitting the regulation of population density below carrying capacity. *American Naturalist,* **96**: 29–33.

MANNING, A., 1959. The sexual behaviour of two sibling *Drosophila* species. *Behaviour,* **15**: 123–145.

NAYLOR, A., 1959. An experimental analysis of dispersal in the flour beetle *Tribolium confusum. Ecology,* **40**: 453–465.

SAKAI, K., T. NARISE, Y. HARAIZUMI, AND S. IYAMA, 1958. Studies on competition in plants and animals. IX. Experimental studies on migration in *D. melanogaster. Evolution,* **12**: 93–101.

SEXTON, O. J., AND H. D. STALKER, 1961. Spacing patterns of female *D. paramelanica. Animal Behaviour,* **9**: 77–81.

WYNNE-EDWARDS, V. C., 1962. *Animal dispersion in relation to social behaviour.* Oliver and Boyd, Edinburgh, 653 pp.

Reference: *Biol. Bull.*, **148**: 286–302. (April, 1975)

EFFECTS OF TEMPERATURE ON THE MORPHOLOGY OF HEMOCYTES AND COAGULATION PROCESS IN THE MOLE-CRAB *EMERITA* (=*HIPPA*) *ASIATICA*

M. H. RAVINDRANATH

Department of Zoology, University of Madras, Chepauk, Madras, Tamil Nadu, 600005, India

It can be seen from previous works that the hemocytes in arthropods described by different workers in this field do not lend themselves for a common classification (see literature cited in Ravindranath, 1973, 1974a, 1974b) to enable comparisons of their structure and functions. Previous workers (Jones, 1962; McLaughlin and Allen, 1965) consider that classification based on morphological features of the cells in question may be more valid than any based on their physiological roles. Different functions may be performed by the same cell and apparently different structural types may be performing similar functions. The classification suggested by Jones (1962) for insect hemocytes on the basis of the structural features was found to be applicable to other groups of arthropods (Ravindranath, 1973, 1974a, 1974b). It would be of interest to know whether such a classification can be extended to the hemocytes of decapod crustaceans, in which there is considerable diversity of views (Halliburton, 1885; Hardy, 1892; Cuénot, 1895; Bruntz, 1905; Kollmann, 1908; Tait and Gunn, 1918; George and Nichols, 1948; Toney, 1958; Dall, 1964; Hearing and Vernick, 1967; Wood and Visentin, 1967; Cheney, 1971; and Johnston, Elder and Davies, 1973).

There is also divergence in the views of authors regarding the roles hemocytes play in gelification or coagulation of plasma in arthropods (Grégoire, 1970). A number of authors (Loeb, 1903; Muttkowski, 1924; Yeager, Shull and Farrar, 1932; Yeager and Knight, 1933; and Beard, 1951), believe that gelification of plasma is initiated by a factor resulting from agglutination of hemocytes. Beard (1951) believes that coagulation can be inhibited by keeping all hemocytes in a dispersed state. On the other hand experiments carried out by Grégoire (1953) indicate that agglutination of cells may not play any part in the hemolymph coagulation. The above author, like many other previous investigators (Hardy, 1892; Tait, 1911), considers that the contents of highly unstable explosive corpuscles with hyaline cytoplasm and eccentric, cart-wheel-like nuclei (corpuscles called coagulocytes or explosive corpuscles or cystocytes) play a decisive role in gelification of hemolymph. While this is so, a number of recent investigators, based on light and electron microscopic observations (George and Nichols, 1948; Dumont, Anderson and Winner, 1966; Hearing and Vernick, 1967; Moran, 1971; and Scharrer, 1972) have attributed the function of hemolymph coagulation to granular hemocytes, which differ morphologically from the explosive corpuscles or cystocytes (see Hardy, 1892; Grégoire, 1970). Although direct evidence is lacking to show that dissolved contents of the granules induce coagulation, Scharrer (1972, p. 313) states that "there remains little doubt that the stepwise transformation of the special cytoplasmic inclusions discussed here, culminating in the release of their content into the hemolymph, play a decisive role in clotting process."

In view of the above findings, an attempt has been made in the present study to classify the hemocytes of *Emerita asiatica* before determining their functional role in coagulation. Such a step may obviate the difficulties faced by previous workers who, on account of the terminology adopted by them, were not clear of the identification of the hemocytes involved in one or the other of the functions of the hemocytes.

MATERIALS AND METHODS

Specimens of the mole-crab *Emerita* (= *Hippa*) *asiatica* were collected at low tides from the shores of Madras Beach, opposite University Campus. Immediately after collection, the specimens were taken to the laboratory and were used for the investigation within six hours. The animals were kept in containers previously filled with sand obtained from the collecting area and were provided with sea water. The size, sex, reproductive and molt cycle stages were recorded prior to collection of blood samples. The stages of the molt cycle were identified using the criteria suggested by Drach (1939).

Blood samples were collected by cutting the first walking leg of the animal. Observations were made on unfixed, fresh preparations as well as on unfixed, stained preparations. The stains used were 0.1% aqueous toluidine blue (BDH 837530) and 0.5% aqueous bromophenol blue. For observations of living cells by phase contrast microscopy, a drop of blood was taken on the glass slide and immediately covered with a cover glass.

Effects of temperature on the morphology of the hemocytes as well as on clotting time were recorded subsequent to immersing the animal in seawater at various temperatures and time following the procedure of Yeager, Shull and Farrar (1932). Initially, the animals were individually kept immersed in sea water for one minute at room temperature. Each animal was taken out, the water was drained and wiped off with filter paper. The region of the leg to be cut was particularly carefully wiped. Care was taken to avoid mixing of sand particles or sea water with the blood sample.

Analyses were carried out in animals immersed for one minute at different temperatures at 5° C intervals from 5 to 45° C. In subsequent experiments the immersion time was prolonged to 2, 5 and 10 minutes. In all these experiments the temperature was maintained in a water bath. Results recorded were based on six experimental animals in each case.

The alterations of hemocytes were recorded after immersion as follows: a stop watch was set at the time of cutting the tip of the leg; the first blood drop was then placed on a clean slide and was covered with a coverslip; the cells were then viewed in phase microscopy under low power, which facilitated viewing the alterations of about 120–200 cells at a time; and the final time was recorded when all the hemocytes in question were degranulated and disintegrated.

The clotting time was determined following the procedure described by Peters and Long (1973). The first drop of hemolymph was placed immediately on a slide and clotting time was determined. The slide was tilted once each 15 seconds, and gelification of plasma was indicated when tilting the slide no longer resulted in conformational change of the drop.

The quick tilt technique of Stewart, Dingle and Odense (1966) was also attempted. In this procedure aggregation and agglutination of hemocytes occurred readily and the plasma gelification was much delayed when compared to the time obtained with the previous method.

RESULTS

General Observations

The hemolymph of *Emerita asiatica* is a clear pale straw-yellow, watery fluid. Occasionally it is colorless. When a drop of hemolymph is allowed to stand at room temperature (28° C), the fluid transforms into a gel in about three minutes. On long standing, the hemocytic meshes embedded in the gel darken. Gelification of plasma is known to be brought about as a result of agglutination of hemocyte or by alterations of fragile hyaline hemocytes (= coagulocytes, = cystocytes) or by distintegration and degranulation of coarse granular hemocytes. Due to paucity of information regarding the morphology and functions of hemocytes of *Emerita asiatica*, the morphology of hemocytes had to be studied.

Morphology of hemocytes

The hemocytes of *Emerita asiatica* can be divided into six types based on their morphology. Each cell type can be easily distinguished from the other types. The hemocytes were classified using the classification and terminology suggested by Jones (1962). The six types of hemocytes recognized in *Emerita asiatica* are prohemocytes, plasmatocytes, granular hemocytes, cystocytes, spherule cells and adipohemocytes. The general characteristics of the hemocytes are presented in Table I. The hemocyte types are compared with those of other decapods in Table II.

Prohemocytes. These are always small, mostly round to ovoid cells, characterized by small amounts of cytoplasm and the nucleus comprising the greater amount of the cell volume (Figs. 1–4, 11). The size of these cells varies from 7 to 11 μ. These cells could easily be seen in heat fixed preparations of male at intermolt, premolt and freshmolt stages. Occasionally, morphological variations are seen among prohemocytes. In some cases, the cytoplasm may be smooth. In a few cases they can be seen with refractile, boat-shaped granules or with refractile spherules. In some prohemocytes, a big vacuole can be observed giving a signet ring appearance to the cells.

Plasmatocytes. These are rare among circulating hemocytes; they are weakly basophilic cells and exceedingly variable in form (Figs. 5, 6). The cytoplasm contains few non-refractile fine orthochromatic granules. Occasionally they send out blunt pseudopodia. The length of these cells varies from 10 to 18 μ.

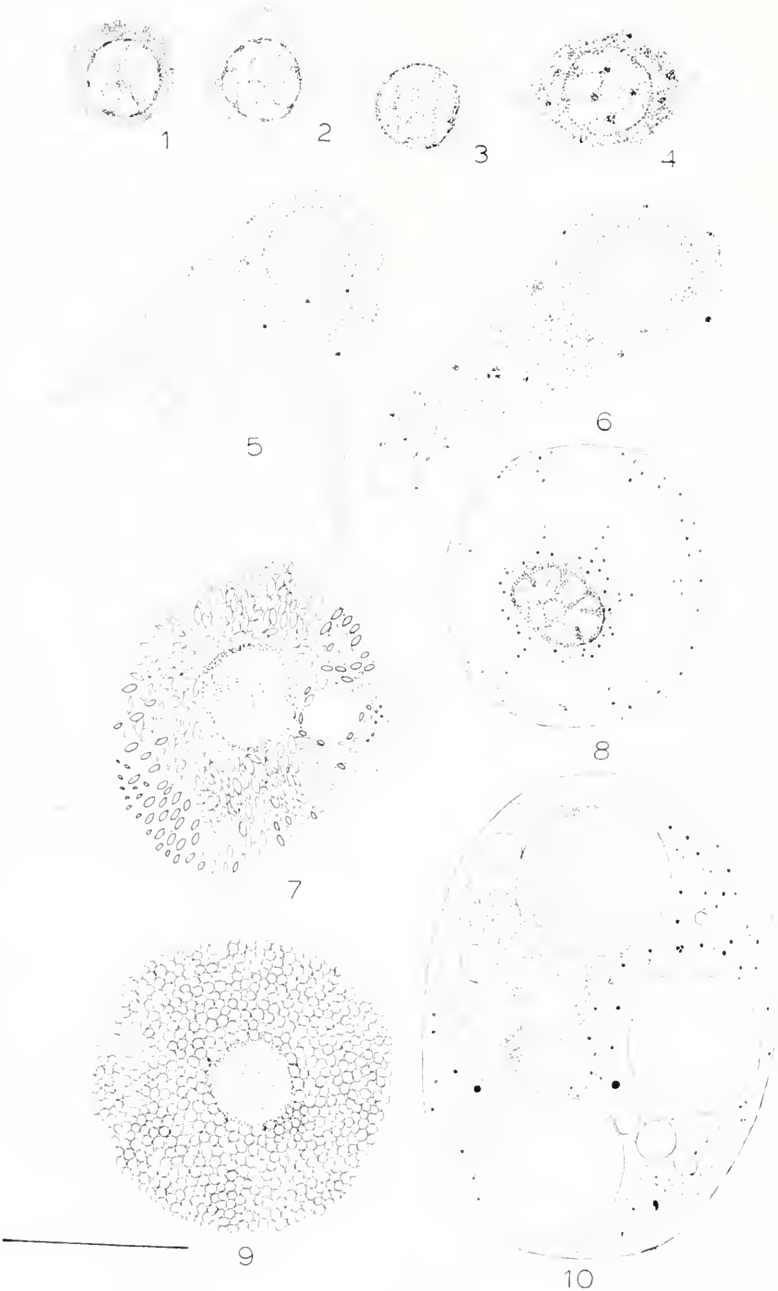
Granular hemocytes. These are basophilic cells, with refractile boat-shaped or elliptical granular inclusions. These cells vary in their length from 12 to 30 μ (Figs. 7, 12). The nucleus is spherical, homogeneous and centrally situated. Its presence is masked by the mass of granules.

The morphology of the granular hemocytes changed significantly when a drop of blood was added to a drop of toluidine blue. The cells swell like balloons, the granules tend to dissolve and become finely granular (Fig. 13). The fine granules accumulated towards the periphery of the cells and exhibited quick jostling move-

TABLE I

The general characters of the hemocyte types of Emerita asiatica.

Characteristics	Prohemocytes	Plasmatocytes	Granular hemocytes	Cystocytes	Spherule cells	Adipohemocytes
Size	7–11 μ	10–18 μ	14–20 μ	15–20 μ	12–22 μ	20–32 μ
Shape	round-oval non-refractile or refractile	oval-spindle polymorphic	oval to polymorphic refractile	oval, non-refractile	round, refractile	oval, refractile
General nature	intensely basophilic, cytoplasm is orthochromatic	basophilic, cytoplasm is orthochromatic in toluidine blue	basophilic, cytoplasma is orthochromatic	weakly basophilic, cytoplasm is orthochromatic	basophilic, cytoplasm is orthochromatic	basophilic, cytoplasma is orthochromatic
Nucleus	homogeneous central	homogeneous central	homogeneous central	heterogeneous, cart-wheel like eccentric	granulated-central	homogeneous-eccentric
Cytoplasmic inclusions	absent or present in various shapes	rare-if present phase-dark, round	boat-shaped, elliptical, refractile	rare, fine granules	spherular	spherular-granular and refractile globular
Behavior	remains same till 1 hr.	changes in shape	degranulates vacuolates and disintegrates, time of alteration varies with temperature	changes soon after exposure	changes very slowly	no change till 1 hr.
In toluidine blue	β	—	(—) or β	α	?	granule: α; spherule; β globule: no color
(In bromophenol blue)						
In bright-field	dark blue	dark blue	green to purple	dark blue	blue	spherule: blue globule: yellow
Under phase	blue	blue	green to purple	blue	purple	spherule: no color globule: yellow



FIGURES 1–10. The appearance of *Emerita* hemocytes under phase contrast. Figures 1–4 show prohemocytes; 2 and 3, vacuolated prohemocytes; 5 and 6, plasmatocytes; 7, a granular hemocyte with characteristic granular inclusions and one vacuole; 8, an ovoid cystocyte with eccentric cart-wheel-like nucleus and vacuolated cytoplasm showing fine granules; 9, a spherule cell with characteristic spherular inclusions; and 10, an adipohemocyte with eccentric nucleus and various sizes of fat-like droplets and inclusions. The scale bar indicates 10 μ.

ments. The cells shrink after some time. The nucleus begins to lose its homogeneity, shape and size (Figs. 14, 15). Occasionally these cells appear to be binucleate.

Cystocytes. These are highly unstable round or oval basophilic cells with eccentric and cart-wheel-like nuclei (Fig. 8). Within a minute of removal of hemolymph, these cells disintegrate and cause glassy veils in the plasma surrounding them. The morphology and the behaviour of cystocytes recall the pattern of coagulation brought about by fragile hyaline hemocytes or coagulocytes of some insects (Grégoire, 1951).

During the alteration of cystocytes, in thin wet films, the nuclei have undergone considerable change. Initially the nucleus of a cystocyte is ellipsoid and homogeneous. Slowly the homogeneity is lost and a fine granular network is seen in the nucleoplasm. After a few minutes, the nucleus becomes round and cart-wheel-like, indicative of nuclear pycnosis and chromatic disaggregation. Associated with the structural modifications, the affinity of the nucleus changes for the reactive groups of the dyes bromophenol blue and toluidine blue. At initial phase, the nucleus stains yellow with bromophenol blue and green with toluidine blue. Finally, it stains blue with the acid dye and purple with the basic dye. The changes in the stainability of the nucleus may signify alterations in the reactive groups of nucleic acids and nuclear proteins.

The orthochromatic cytoplasm of cystocytes contains fine, refractile and β-metachromatic granules, which were in continuous movement. These cells range 15 to 20 μ in size.

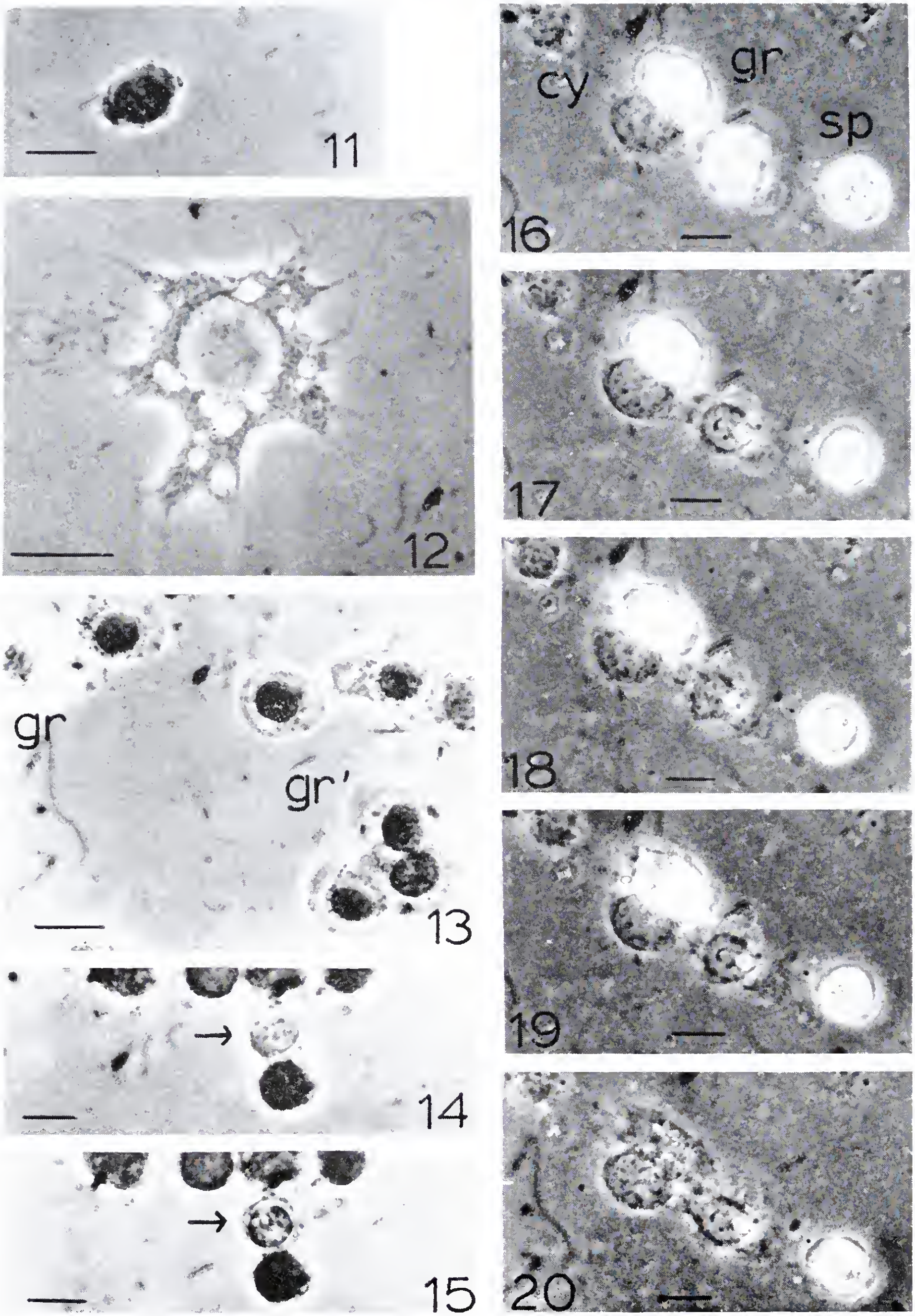
Spherule cells. These are round, refractile basophilic hemocytes with many distinct, uniformly round, acidophilic, refractile spherules (Figs. 9, 16–20). They range in their width from 12 to 22 μ. They do not disintegrate or breakdown into intensely hyaline forms in thin wet films, but after prolonged exposures, some cells become vacuolated and in some, the refractile spherules fuse together (Fig. 20). They do not anastomize to form plasmodia.

The spherules stain dark blue with bromophenol blue. But in phase microscopy, the spherules, soon after adding the dye, show a green color which subsequently become purple. The immobile nature of the spherules and their affinity to aqueous bromophenol blue distinguish this cell type from other hemocytes. This cell type is found in greater numbers only in postmolt period.

Adipohemocytes. On rare occasions, large oval basophilic hemocytes with eccentric nucleus and many refractile droplets or globules of various sizes have been observed in the hemolymph of *Emerita asiatica* (Fig. 10). They have the appearance of small fat-body cells in insects. They measure in length about 20–32 μ.

Observations made on hemocytes of various size groups, sexes, molting and reproductive stages of *Emerita asiatica* reveal that granular hemocytes and cystocytes are the predominant cell types. The percentage of granular hemocytes in fresh preparations varies from 60 to 75%, whereas that of cystocytes varies from 20 to 35%. Other cell types, though rare, are driven into circulation during heat-fixation. No dividing cells were observed among any of the cell types.

When the hemolymph is observed soon after collection, the explosive nature of the cystocytes can be observed. They cause glassy veils in which other cells may get attached. It was noted that the cystocytes are not the only cell type to undergo changes; the granular hemocytes do likewise.


11
12
gr
gr'
13
14
15
cy
gr
sp
16
17
18
19
20

FIGURES 11–20. Photomicrographs of *Emerita* hemocytes. Figure 11 shows a prohemocyte, note the size and cytoplasm: nucleus ratio. The scale bar indicates 10 μ. Figure 12 shows a hemocyte after prechilling, note the well-spread granular hemocyte with ellipsoid granules and

Events associated with coagulation

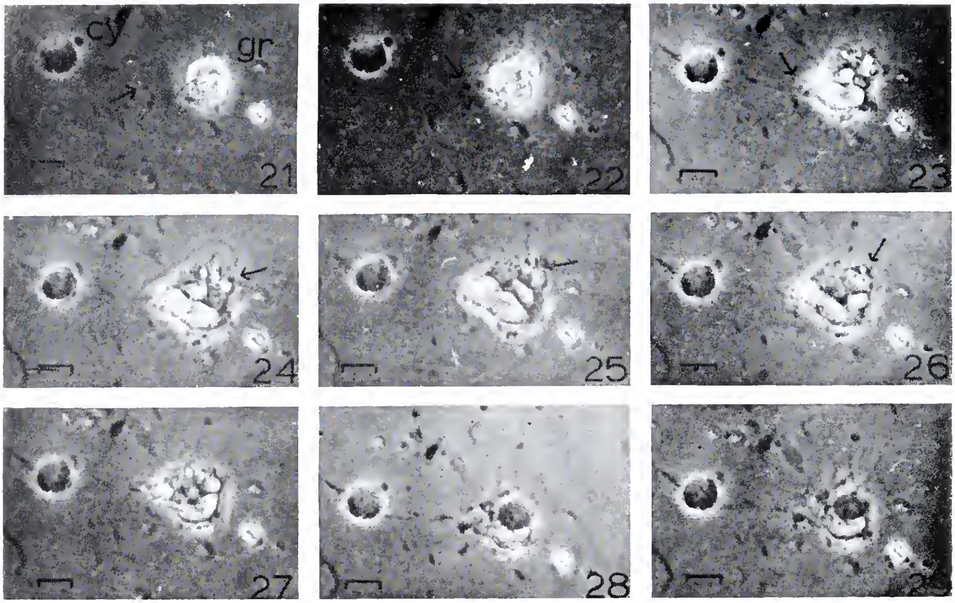
Alterations in granular hemocytes. Figures 13–15, 16–20 and 21–29 show the alterations undergone by granular hemocytes in thin wet films and in stained preparations. Individually these cells exhibit "atypical amoeboid motion" similar to that exhibited by the granular hemocytes of an insect *Blaberus giganteus* (Arnold, 1959, 1961). This process involves the separation of the cell's cytoplasm into an outer hyaline ectoplasm and an inner refractile granular endoplasm. Movement is initially accomplished by the flow of cytoplasm in the region of ectoplasm. The flow of cytoplasm thus results in formation of blunt pseudopodia with scalloped edges. The granular endoplasm did not stream or enter the pseudopodia but some turbulence of tension seemed to occur there. It merely followed the advancing hyaline ectoplasm and conformed to its outlines. These outlines became modified. The base broadened with the inflow of endoplasm, the pseudopodium became lamellar, and subsequently changed completely to the typical amorphous streaming pseudopodium. Under these conditions, vacuoles appeared in the center of granular endoplasm and the boat-shaped granules began to lose their refractility (Figs. 21–23). Figure 12 reveals a granular hemocyte at this stage with numerous pseudopodia and refractile vacuoles. As they lose their refractility, the edges of these inclusions are sharp black and the enclosed granular space bright.

The slow cytoplasmic movements in the granular hemocytes are associated with haphazard jostling of the granules within the small areas of the cells. The surface area of the cell begins to expand to thrice the original cell size. Few granules that have reached the ectoplasm, continually move in and out of it in a shuttling motion. In the ectoplasm, the granules circled irregularly as individuals or as small chains that percolated in and out through an apparent meshwork of relatively stationary granules, which themselves occasionally joined in the action. The shuttling granules slowly dissolved in the ectoplasm and disappeared. The enlarging vacuoles pushed the endoplasmic granules to the periphery of the cells, where they dissolved quickly (Figs. 24–27). The contents of the vacuoles were liberated into the plasma (Figs. 27–29), finally leaving the cytoplasmic meshwork (Fig. 29). This facilitated the interlinking of hemocytes and the agglutination of cells of the same class.

A feature of interest in the alteration of granular hemocyte is that it is initiated quickly in cells which are in the vicinity of bursting cystocytes (Figs. 16–20). The elastic fiber (resulting from bursting of a cystocyte) along with a phase dark granule liberated from another cystocyte were observed to reach a granular hemocyte (Figs. 21–23) prior to the commencement of alterations in the latter.

---

vacuoles, and the fine pseudopodial projections. While the granules have lost their refractility, the vacuoles retain theirs. The scale bar indicates 10 μ. Figure 13 shows granular hemocytes in toluidine blue. The granules, while refractile, do not take up the stain (gr); note the balloon-shaped appearance of the cell and the dissolving granules (gr′). The scale bar indicates 10 μ. In Figures 14 and 15, note the changing shape and staining affinity of the nucleus of the shrunken granular hemocyte. The scale bars indicate 10 μ. Figures 16 through 20 show the alterations of granular hemocytes in the vicinity of burst cystocytes (after immersion of the animal in sea water at 35° C for five minutes). The time interval between each exposure is one minute. Note: cy, cystocytes; gr, granular hemocyte; and sp, spherule cells. The change occurred in the spherule cell in Figure 20. The scale bars indicate 10 μ.



FIGURES 21–29. Photomicrographs of the stepwise alterations in the granular hemocytes (after immersion of the animal in the sea water at 10° C for one minute). The time interval between each exposure is two minutes. Note: cy, cystocyte; and gr, granular hemocyte. The different figures show vacuolization, loss of refractility of granules, dissolution of granules, enlargement and fusion of vacuoles, liberation of vacuolar contents into plasma, and disintegration and anastomosis of cytoplasm. Note a cytoplasmic fiber arising from the cystocyte connecting to the granular hemocyte. The arrows in Figures 21, 22, and 23 show the movement of a phase dark granule in the fiber. The granule from the cystocyte moves and reaches the granular hemocyte. The arrows in Figures 24, 25, and 26 show the jostling granules after losing their refractility. Note their dissolution in the cytoplasm, from which they do not emerge. The scale bars indicate 10 μ.

Similar alterations in the granular hemocytes were also observed in conglomerates of the hemocytes. While complete alterations were observed in the cells found in the periphery, the cells inside the clusters showed different stages of incompleteness. In some cells, a sudden stoppage of the movement of granules was observed while, in some others, the granules remained refractile for more than an hour. Similarly, Grégoire (1970, Fig. 14) observed no distintegration or degranulation in the clusters of granular hemocytes of *Limulus polyphemus.* The sudden stoppage in the movement of granules calls to the mind the observations of Harvey (1942) on the movement of granules during cyclosis in the cells of *Nitella.* He observed that any sudden decrease or increase in temperature caused a shock stoppage of the movement of granules.

Effect of temperature on alterations of granular hemocytes. To determine whether the alterations in the granular hemocytes lead to coagulation of the hemolymph, attempts were made to investigate whether the factors known to influence the coagulation also affect the alterations in the hemocytes. One such factor that is known to influence clotting time is temperature (Dean and Vernberg, 1966).

TABLE II

The classification and terminology of hemocytes of Emerita asiatica *compared with those of other decapods.*

Present study	Hardy, 1892	Cuenot, 1895	Kollmann, 1908	Tait and Gunn, 1918	George and Nichols, 1948	Toney, 1958	Dall, 1964	Wood and Visentin, 1967
Emerita asiatica	*Astacus fluviatilis*	*Astacus fluviatilis*	List A	*Astacus fluviatilis*	List B	*Cambarus sp; Callinectus sapidus.*	*Metapanaeus mastersii*	*Orconectus virilis*
Prohemocyte	—	amibocyte I	hyaline leucocyte I	—	lymphoid cell (Type-I)	lymphoid cell or monocyte	lymphocyte	hyaline cell I
Plasmatocyte	—	amibocyte II	hyaline leucocyte II	thigmocyte	colorless, semihyaline thigmotoctic amoebocyte (Type-II)	—	thigmocyte	hyaline cell II
Granular hemocyte	eosinophil corpuscle	amibocyte à granulations acidophiles (III)	leucocyte granules Pl.II (21,22)	eosinophil, granular amoebocyte	cells with coarse refractile acidophilic granule (Type-III & IV)	explosive refractive granulocyte	large granular amoebocyte	granulocyte.
Cystocyte	explosive corpuscle	amibocyte-II (degenerative)	leucocyte (degenerative) Pl.II(ii)	explosive corpuscle	—	explosive refractive granulocyte	—	—
Spherule cell	basophil	cellules proteiques	cellules spherules	—	Type III of *Cambarus bastoni*	—	—	—
Adipohemocyte	—	—	—	—	—	—	—	—

List A: *Astacus fluviatilis, Palemonetes varians, Palemon serratus, Gebia deltura, Maia squinado, Pisa tetraodon, Carcinus maenas, Portunus depurator, Portunus puber, Corystes cassirelaunus, Pagurus bernhardus, Dromia vulgaris, Galathea intermedia, Galathea squamifera.*
List B: *Libinia dubia, Uca pugnax, Hippa talpoida, Panopius herbstii, Callinectes sapidus, Cambarus bastoni.*

The effect of temperature on the alterations of granular hemocytes is shown in the graph (Fig. 30). The time interval between exposure of blood and complete disintegration of granular hemocytes is recorded after immersion in a seawater bath at the various temperatures and intervals referred to earlier. The cells are fixed after one-minute immersion at 45° C. Lowering of the temperature from 15° C to 5° C results in progressive delay of the alterations of granular hemocytes. On the other hand, the rate of alterations was quickened progressively with increasing temperature. The trend in the temperature-dependent alterations of granular hemocytes remained more or less the same irrespective of the time interval of immersion. But, the time taken for alteration at a particular temperature decreased with increasing immersion time up to five minutes. The delay in the alteration of granular hemocytes at a particular temperature after one-minute immersion

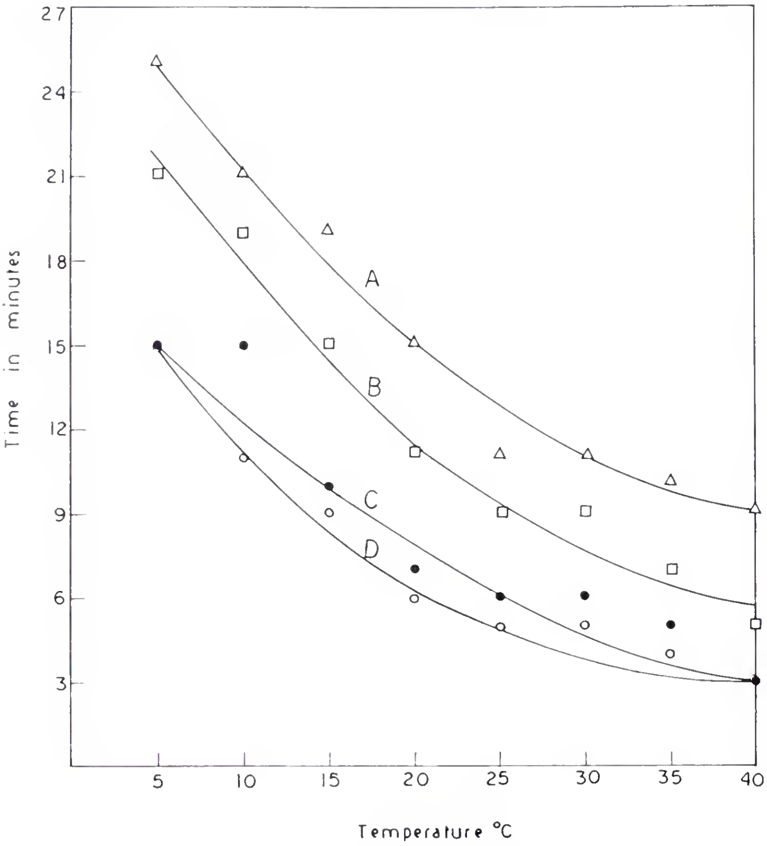


FIGURE 30. Shows the time taken for alteration of the granular hemocyte at different temperatures; observations were made on blood samples collected after immersing the animal in sea water at different time intervals. Triangles (A) indicate immersions of one minute; squares (B), of two minutes; dark circles (C), of five minutes; open circles (D), of ten minutes.

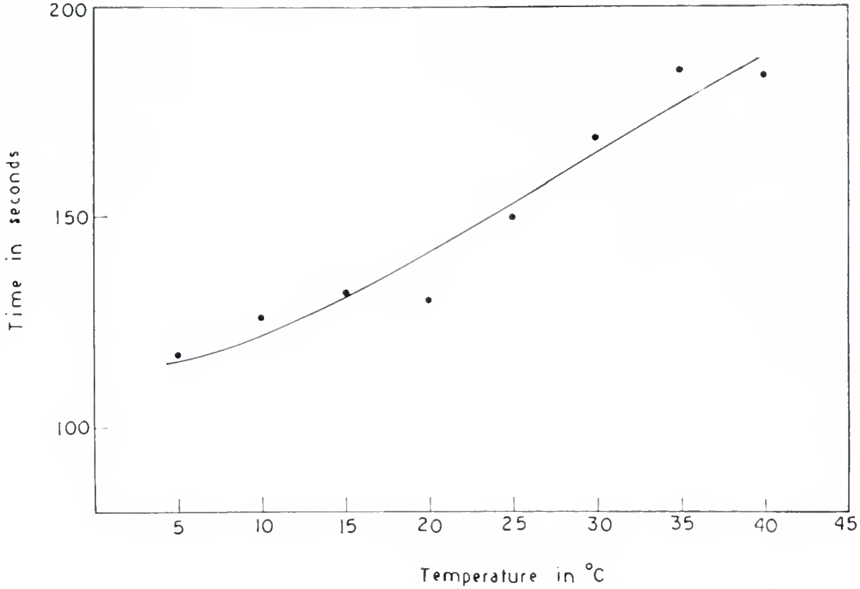


FIGURE 31. Shows the time taken for gelification of plasma at different temperatures. Observations were made on blood samples collected after immersing the animal in sea water for five minutes.

probably reflects the shock response of the animal to sudden change in the temperature of the environment. The animal appears to recover from the shock within five to ten minutes, for there is no significant dfference in the time taken for the alteration at any temperature after five- and ten-minute immersions.

On the contrary, the clotting times recorded at different temperatures show an opposite trend (Fig. 31). It was noted that the times taken for gelification of plasma increase with temperature, and here the agglutination of hemocytes did not precede plasma gelification.

DISCUSSION

The observations reported in the present study on the hemocytes of *Emerita asiatica* solve a long standing confusion in the nomenclature of decapod crustaceans (Table II) and enable comparison of different types of hemocytes of crustaceans with the hemocyte types of other arthropods. Absence of mitotically dividing cells in the plasma, occurrence of differences among prohemocytes and the wide size range of different major hemocyte types support the view put forward earlier (Ravindranath, 1973, 1974a, 1974b, 1974c) that prohemocytes may be the progenitors of the various major cell classes.

One of the striking features in the composition of the hemocytes of *Emerita asiatica* is the predominance of one cell type, the granular hemocyte. Such a feature is not uncommon among decapods and king-crabs (see Schulz 1925; George and Nichols, 1948; Dumont, Anderson and Winner, 1966). An interesting aspect of the granular hemocyte is the nature of granular inclusions, which are refractile and boat-shaped. Such inclusions are also present in the granular hemocytes in insects, *Carausius morosus* (Millara, 1947, Pl.III Figs. 2 and 3), *Locusta migratoria* (Hoffmann, 1967), *Blaberus discoidalis*, *B. giganteus* and *Leucophaea maderae* (Arnold, 1972, Figs. 105 and 123); and also in *Peripatus sp.* (Grégoire, 1955, Figs. 5 and 6); in chilopods, *Lithobius forficatus* (Grégoire, 1970); arachnids, *Limulus polyphemus* (Grégoire, 1955, Figs. 7 and 14; Dumont, Anderson and Winner, 1966); and in several species of scorpions (Kollmann, 1908; Ravindranath, 1974a). But such a cell type does not fall into any one of the categories of hemocytes classified by Jones (1962).

Another feature of interest is that the prohemocytes, plasmatocytes, spherule cells and adipohemocytes are rare in fresh preparations but common in heat-fixed preparations. Jones (1962) has suggested that this method of preparation drives the hemocytes into circulation from the sites of their accumulation. It is also possible that this method may alter the morphology of cell types.

It is known that the clotting or coagulation of the blood of arthropods consists of two physiologically distinct processes which can occur independently or together: initially hemocyte agglutination or cell coagulation occurs and this may be followed by gelification of the plasma, termed plasma coagulation (Grégoire and Tagnon, 1962). In *Emerita asiatica*, the hemocyte coagulation and plasma gelification occur independently. The hemocytes remain in a dispersed state when plasma gelification has occurred. This finding derives support from the observations of Grégoire (1953). In insects, he suggested that gelification of plasma is initiated by cystocytes. The observations made here and by Jones (1962) support this view. They reveal that cystocytes undergo changes within a minute or two of exposure, irrespective of the temperature differences. Similar findings were reported in the works of Hardy (1892) and Grégoire and Tagnon (1962).

The plasma gelifies swiftly at colder temperatures, a finding which closely parallels the reports of Peters and Long (1973) but which is in contradiction to the results of Dean and Vernberg (1966) and Joshua, Fischl, Henig, Ishay and Gitter (1973). This trend in gelification in relation to different temperatures is quite opposite to that of the alterations of granular hemocytes.

These findings are in accordance with those of Hardy (1892, p. 170), who observed in *Astacus* that "the eosinophil cells (or granular hemocytes) remained unchanged and alive for a considerable time after the blood has clotted." In the same animal, Tait and Gunn (1918) have also reported that no coagulation follows the cytolysis of the eosinophil amoebocytes (= granular hemocytes) which occurs after cytolysis of explosive corpuscles (=cystocytes). Grégoire (1970) likewise reported that gelification occurred when all the hemocytes (except cystocytes) had still retained most of their granules intact. All these observations cast doubt on the direct role of granular hemocytes in coagulation.

The stepwise alterations of the granular hemocytes lead to cellular agglutination or plasmodial formation. This kind of cellular agglutination is an independent process in *E. asiatica* and does not lead to plasma gelification, but takes place subsequent to it. Hemocyte agglutination was reported in other crustaceans (see Table I of Grégoire and Tagnon, 1962) and also in *Limulus polyphemus* (Loeb, 1903;

Copley, 1947; Kenney, Belamarich and Shepro, 1972). All these authors have shown that low temperatures retard the aggregation and agglutination of *Limulus* granular hemocytes. Kenney *et al.* (1972) have suggested that the aggregation-promoting factor is heat-labile. Similarly the temperature-dependent alterations of granular hemocytes of *Emerita asiatica* suggest that the factor promoting the alterations of the granular hemocytes may also be heat-labile. Further, the observations made in the present study (Figs. 21–22) indicate that the factor in question may be liberated from cystocytes. In this regard the observation, that the first signs of alteration of granular hemocytes occur only in the vicinity of cystocytes, is of considerable significance.

It appears in the light of the present investigation that cystocytes may perform two functions: (1) they promote plasma gelification, and (2) they initiate the stepwise transformation of granular hemocytes, which in turn leads to agglutination of cells and to the formation of a meshed network. The significance of the dissolution of the granules is not clear at present.

Another interesting feature observed in the present study is the similarity in the changing pattern of nuclei of both cystocytes and granular hemocytes. A strikingly parallel feature was reported among the explosive corpuscles (= cystocytes) and eosinophil corpuscles (= granular hemocytes) of *Astacus* by Hardy (1892, p. 169), who observed that "the nucleus (of eosinophil corpuscle) comes into view and acquires that intense distinctness which we noticed as such a remarkable features of the rigor mortis, or clotting, of the nuclei of the explosive corpuscles." Dumont, Anderson and Winner (1966) traced the changes that take place in the nucleus ultrastructurally. Although the significance of the changes that occur in the nucleus of these cell types is not clear, the similarity in the behaviour of the nuclei in both these cell types suggests an ontogenic relationship between them.

I am greatly indebted to Professors Dr. G. Krishnan and Dr. K. Ramalingam and to Drs. M. H. Rajeswari-Ravindranath and T. Subramoniam for reading the manuscript and making many valuable suggestions for its improvement. Sincere appreciation is expressed to Dr. S. Raghukumar for taking practically all the photographs upon which the supporting observations were based. I am also thankful to Professor Dr. C. V. Subramaniam, Professor of Botany, for extending necessary facilities.

SUMMARY

1. The hemocytes of *Emerita asiatica* have been studied in fresh preparations by phase contrast microscopy and also after staining.

2. With phase contrast microscopy, the following categories of hemocytes can be identified: (a) nondividing prohemocytes, (b) pleomorphic plasmatocytes, (c) intact and altering granular hemocytes, (d) quickly lysing cystocytes, (e) intact spherule cells, and (f) adipohemocytes.

3. This classification and terminology solve a long standing confusion in the nomenclature of hemocytes of decapods and enable comparisons between the different hemocyte types of crustaceans and the hemocyte types of other arthropods to be made.

4. Cystocytes and granular hemocytes constitute more than 95% of the hemocytes.

5. In thin wet films, the granular hemocytes undergo alterations which include loss of shape, refractility and granules, followed by vacuolization and disintegration leading to agglutination of the cells.

6. The alterations of the granular hemocytes are temperature-dependent, and lowering of the temperature results in progressive delay of the alterations.

7. There is no correlation between alteration of granular hemocytes and clotting time at different temperatures. At lower temperatures, gelification of plasma occurs when the granular hemocytes remain unaltered. The observations reveal that the granular hemocytes may not play any role in gelification of plasma.

8. Further, the process of cellular agglutination does not play any part in the phenomenon of plasma gelification, unlike this process in insects as reported by Grégoire.

9. Cystocytes, which have been referred to as explosive corpuscles by previous crustacean workers, disintegrate within a minute or two of exposure at all temperatures. Evidence supports their role in plasma gelification.

10. The observations also indicate that the substances liberated from the cystocytes initiate the stepwise transformation of granular hemocytes.

LITERATURE CITED

Arnold, J. W., 1959. Observations on amoeboid motion of living haemocytes in the wing veins of *Blaberus giganteus* (L) (Orthoptera: Blattidae). *Can. J. Zool.*, **37**: 371–375.

Arnold, J. W., 1961. Further observations on amoeboid haemocytes in *Blaberus giganteus* (L) (Orthoptera: Blattidae). *Can. J. Zool.*, **39**: 755–766.

Arnold, J. W., 1972. A comparative study of the haemocytes (blood cells) of cockroaches (Insecta: Dictyoptera: Blattaria), with a view of their significance in taxonomy. *Canad. Ent.*, **104**: 309–348.

Beard, R. L., 1951. Experimental observations on coagulation of insect hemolymph. *Physiol. Zool.*, **23**: 47–57.

Bruntz, L., 1905. Etudes physiologiques sur les phyllopodes branchipiodes. Phagocytose et excretion. *Arch. Zool. Exp. Gen.*, **76**: 1–67.

Cheney, D. P., 1971. A summary of invertebrate leucocyte morphology with emphasis on blood elements of the Manila Clam, *Tapes semidecussata*. *Biol. Bull.*, **140**: 353–368.

Copley, A. F. L., 1947. The clotting of *Limulus* blood. *Fed. Proc.*, **6**: 90–91.

Cuenot, L., 1895. Etudes physiologiques sur les crustacees decapodes. *Arch. Biol. Liege.*, **13**: 245–303.

Dall, W., 1964. Studies on the physiology of shrimp, *Metapenaeus mastersii* (Haswell) (Crustacea: Decapoda: Penaeidae). I. Blood Constituents. *Aust. J. Mar. Freshw. Res.*, **15**: 145–61.

Dean, J. M., and F. J. Vernberg, 1966. Hypothermia and blood of crabs. *Comp. Biochem. Physiol.*, **17**: 19–22.

Drach, P., 1939. Mue et cycle d'intermue chez les crustaces decapodes. *Ann. Inst. Oceanogr.*, **19**: 103–391.

Dumont, J. N., E. Anderson, and G. Winner, 1966. Some cytologic characteristics of the hemocytes of *Limulus* during clotting. *J. Morphol.*, **119**: 181–217.

George, W. G., and J. Nichols, 1948. A study of the blood of some crustacea. *J. Morphol.*, **83**: 425–443.

Grégoire, C., 1951. Blood coagulation in arthropods. II. Phase contrast microscopic observations on hemolymph coagulation in sixty-one species of insects. *Blood*, **6**: 1173–1198.

Grégoire, C., 1953. Blood coagulation in arthropods. III. Reactions of insect hemolymph to coagulation inhibitors of vertebrate blood. *Biol. Bull.*, **104**: 372–393.

Grégoire, C., 1955. Blood coagulation in arthropods. VI. A study by phase contrast microscopy of blood reactions in vitro in Onychopora and in various groups of arthropods. *Arch. Biol. Paris*, **66**: 489–508.

Grégoire, C., 1970. Haemolymph coagulation in arthropods. *Symp. Zool. Soc. London.*, **27**: 45–74.

Grégoire, C., and H. J. Tagnon, 1962. Blood coagulation. Pages 435–482 in M. Florkin and H. S. Mason, Eds., *Comparative Biochemistry*, Volume 4. Academic Press, New York and London.

Halliburton, W. E., 1885. On the blood of the decapod crustacea. *J. Physiol.*, **6**: 300–335.

Hardy, W. B., 1892. The blood corpuscles of the crustacea, together with a suggestion as to the origin of the crustacean fibrin-ferment. *J. Physiol.*, **13**: 165–190.

Harvey, E. N., 1942. Hydrostatic pressure and temperature in relation to stimulation and cyclosis in *Nitella flenilis*. *J. Gen. Physiol.*, **25**: 855–863.

Hearing, V., and S. H. Vernick, 1967. Fine structure of the blood cells of the lobster *Homarus americanus*. *Chesapeake Sci.*, **8**: 170–186.

Hoffmann, J. A., 1967. Etude des hemocytes de *Locusta migratoria* L. (Orthoptere). *Arch. Zool. Exp. Gen.*, **108**: 251–291.

Johnston, M. A., H. Y. Elder, and P. S. Davies, 1973. Cytology of *Carcinus* haemocytes and their function in carbohydrate metabolism. *Comp. Biochem. Physiol.*, **46A**: 569–582.

Jones, J. C., 1962. Current concepts concerning insect hemocytes. *Amer. Zool.*, **2**: 209–246.

Joshua, H. J., F. B. Fischl, E. Hening, J. Ishay, and S. Gitter, 1973. Cytological biochemical and bacteriological data of the hemolymph of *Vespa orientalis*. *Comp. Biochem. Physiol.*, **45B**: 167–175.

Kenny, D. M., F. A. Belamarich, and D. Shepro, 1972. Aggregation of horseshoe crab (*Limulus polyphemus*) amoebocytes and reversible inhibition of aggregation by EDTA. *Biol. Bull.*, **143**: 548–567.

Kollmann, M., 1908. Recherches sur les leucocytes et le tissue lympoide des invertebres. *Ann. Sci. Natur. Zool.*, **8**: 1–238.

Loeb, L., 1903. On the coagulation of the blood of some arthropods and on the influence of pressure and traction of the protoplasm of the blood cells of arthropods. *Biol. Bull.*, **3**: 301–318.

McLaughlin, R. E., and G. Allen, 1965. Description of hemocytes and the coagulation process in the Boll Weevil, *Anthonomus grandis* Boheman (curculionidae). *Biol. Bull.*, **128**: 112–124.

Millara, P., 1947. Contribution a l'etude cytologique et physiologique des leucocytes d'insects. *Bull. Biol. France and Belgium*, **81**: 129–153.

Muttkowski, R. A., 1924. Studies on the blood of insects. III. The coagulation and clotting of insect blood. *Bull. Brooklyn Entom. Soc.*, **19**: 128–144.

Moran, D. T., 1971. The fine structure of cockroach blood cells. *Tissue Cell*, **3**: 413–422.

Peters, R. I., and G. E. Long, 1973. Some alterations in hemolymph proteins of *Hemigrapsus nudus* associated with temperature acclimation. *Comp. Biochem. Physiol.*, **46A**: 207–213.

Ravindranath, M. H., 1973. The hemocytes of a millipede, *Thyropygus poseidon*. *J. Morphol.*, **141**: 257–268.

Ravindranath, M. H., 1974a. The hemocytes of a scorpion, *Palamneus swammerdami*. *J. Morphol.*, **144**: 1–10.

Ravindranath, M. H., 1974b. The hemocytes of an isopod, *Ligia exotica* Roux. *J. Morphol.*, **144**: 11–22.

Ravindranath, M. H., 1974c. Changes in the population of circulating haemocytes during moultcycle phases of the millipede, *Thyropygus poseidon*. *Physiol. Zool.*, in press.

Scharrer, B., 1972. Cytophysiological features of hemocytes in cockroachs. *Z. Zellforsch.*, **129**: 301–319.

Schulz, N. F., 1925. Crustacean. Pages 669–746 in H. Winterstein, Ed., Handbuch der vergleichenden physiologie, 1(1): Physiologie der Korperslifte. Gustav Fischer, Jena.

Stewart, J. E., J. R. Dingle, and P. H. Odense, 1966. Constituents of the hemolymph of the lobster *Homarus americanus* Milne Edwards. *Can. J. Biochem.*, **44**: 1447–1459.

TAIT, J., 1911. Types of crustacean blood coagulation. *J. Mar. Biol. Ass. U.K.* **9**: 191–198.

TAIT, J., AND J. D. GUNN, 1918. The blood of *Astacus fluviatilis:* a study in crustacean blood with special reference to coagulation and phagocytosis. *Quart. J. Exp. Physiol.*, **12**: 5–80.

TONEY, M. E., JR., 1958. Morphology of the blood cells of some crustacea. *Growth,* **22**: 35–50.

WOOD, P. J., AND L. P. VISENTIN, 1967. Histological and histochemical observations of the hemolymph cells in the crayfish, *Orconectes virilis. J. Morphol.,* **123**: 559–568.

YEAGER, J. F., AND H. H. KNIGHT, 1933. Microscopic observations on blood coagulation in several species of insects. *Ann. Entomol. Soc. Amer.,* **26**: 591–602.

YEAGER, J. F., W. E. SHULL, AND M. D. FARRAR, 1932. On the coagulation of blood from the cockroach, *Periplaneta orientalis* (Linn.) with special reference to blood smears. *Iowa State Coll. J. Sci.,* **6**: 325–346.

Reference: *Biol. Bull.*, **148**: 303–319. (April 1975)

OSMOTIC CONSTITUENTS OF THE BLOOD PLASMA AND PARIETAL MUSCLE OF *SQUALUS ACANTHIAS* L.

JAMES D. ROBERTSON

Zoology Department, University of Glasgow, Glasgow, Scotland U.K., and Dunstaffnage Marine Research Laboratory, Oban, Scotland

The blood plasma of elasmobranchs is isosmotic or slightly hyperosmotic to sea water, but ions account for only part of its osmotic concentration, a large fraction being made up by urea and trimethylamine oxide (Holmes and Donaldson, 1969). These nitrogenous compounds are also present in high concentration in muscle (Smith, 1929; Dyer, 1952).

In this paper an attempt is made to outline the sea water-plasma and plasma-muscle steady states by comprehensive analyses of muscle and plasma of specimens of the spiny dogfish *Squalus acanthias*. Measurements of osmotic concentration of plasma and muscle have been made and compared with the sum of analyzed constituents, values of the latter, first obtained as milligram-ion or millimolar concentrations per kilogram solvent water, being converted to milliosmoles by the appropriate osmotic coefficients. Imprecision arises here owing to lack of knowledge of some coefficients, and because of the possibility that some of the constituents may be bound to protein, exerting little osmotic effect. Some idea of the amount of ion-binding in muscle has been obtained by analyses of the juice expressed from muscle by a tissue press or obtained by ultracentrifugation. Estimates have also been made of the extracellular space in muscle, thus enabling intracellular concentrations to be calculated.

MATERIALS AND METHODS

Specimens of *Squalus acanthias* were caught by trawl in the Firth of Clyde and were kept in tanks of flowing sea water. Salinity during several summer periods varied from 32.2–33.4‰ (18.20–18.88 g Cl/liter) with temperatures of 9–12° C. After stunning the fish, blood was withdrawn by syringe or pipette under liquid paraffin from the heart and placed in centrifuge tubes kept in a beaker of crushed ice. After centrifugation, the plasma, still under paraffin, was removed and used for analysis. Samples of white parietal muscle were taken from the dorsal and lateral region of the tail just behind the second dorsal fin (epaxial muscles). After light blotting with filter paper, separate samples were used for cations (ashing at 550° C in the presence of sulphuric acid), determination of dry weight and the preparation of trichloroacetic extracts, tungstic acid extracts and zinc hydroxide extracts.

Methods for the analysis of Na, K, Ca, Cl and SO_4 of muscle and plasma were essentially those of Robertson (1949, 1960) with appropriate modifications for the lower concentration of ions in elasmobranchs. Magnesium was estimated by Heagy's (1948) method. Some of the analyses for Ca were done by atomic absorption spectrophotometry; interference by phosphate in the Ca estimation of muscle and muscle juice was suppressed by lanthanum chloride at a final concentration in the solution investigated of 1% La ions. Lactate of plasma and muscle was determined in trichloroacetic acid filtrates (Hullin and Noble, 1953).

Ice-cold trichloroacetic acid muscle filtrates were used immediately for phosphate fractionation (Umbreit, Burris and Stauffer, 1949), the phosphorus of the fractions being determined according to Sumner (1944), and for determination of free and bound creatine (Ennor, 1957). TCA filtrates were also used for analysis of betaine and trimethylamine oxide (Kermack, Lees and Wood, 1955). For betaine gravimetric estimation of the mixed reineckates of those two compounds was substituted for colorimetry.

This was done in porosity 4 filter crucibles closed with rubber bungs. McIlvaine's buffer solution of pH 2.2 was saturated with ammonium reineckate, and further saturated with the salts of the two compounds by adding a little betaine-HCl and TMAO-HCl in solution. Twenty ml of the filtered solution were used, to which was added 1–2 ml of the muscle or plasma filtrate. After mechanical stirring for 5–6 minutes, the crucibles were left covered for 60 minutes at room temperature. After filtration the precipitate was washed thoroughly with n-propanol saturated with betaine and TMAO reineckates; in the first washing the precipitate was suspended and stirred with about 15 ml of the washing solution, the crucible being closed. Finally, the precipitate was dried with ether and the crucible weighed after 20 minutes in a desiccator. From the previously determined TMAO content of the filtrate and the molecular weight of TMAO-reineckate, 393.49, the amount of this compound in the precipitate is found. Any excess of precipitate is taken to be betaine reineckate, molecular weight 435.53, from which betaine is calculated.

In the TMAO analysis formaldehyde was added to hold back ammonia during the micro-diffusion of the trimethylamine. Tungstic acid filtrates were used in the estimation of the α-amino N of the free amino acids of muscle and plasma (Frame, Russell and Wilhelmi, 1943; Russell, 1945), and of creatinine of muscle and plasma (Owen, Iggo, Scandrett and Stewart, 1954).

Micro-diffusion techniques (Conway, 1962) were used in estimating bicarbonate and ammonium ions, urea (after urease) and total nonprotein N (after micro-Kjeldahl). Tungstic acid filtrates were used for muscle and for the NPN of plasma, but the two ions and urea of plasma were analyzed directly. Zinc hydroxide filtrates were used in the determination of glucose (glucose-oxidase method with Boehringer's Biochemica Test reagents) and of glycerol (Lambert and Neish, 1950).

Water contents were obtained by measuring the difference between fresh and dry weights, plasma samples being dried for 4 hr and muscle for 24 hr at 100–101° C.

A few measurements of extracellular space in muscle were obtained by injecting a solution of inulin in sea water intraperitoneally and taking samples of blood and muscle 14–22 hr afterwards. Inulin was estimated by measuring the fructose obtained after hydrolysis (Roe, Epstein and Goldstein, 1949).

The Krogh-Baldes pressure method (Krogh, 1939, p. 211) was used to measure total concentration (milliosmoles) of plasma and muscle juice, the juice being obtained with a small tissue-press (Krogh, 1938). Most measurements of the juice were made within 1.25 hr of removing the muscle, thus minimizing changes due to breakdown of labile compounds. The osmotic concentration of the plasma and sea water was determined in specimens which had been equilibrated for 24 hr in water of constant chloride content.

Some analyses of ion were made on the muscle juice, and on fluid centrifuged from muscle. Centrifuging was done at 40,000 g (18,000 rpm) for 30–40 minutes at 0° C in polypropylene tubes. From 20 g muscle 5–6 g of centrifuged fluid of water content 88–89% could be obtained, leaving the residual muscle with about 68–70% water.

The following osmotic equivalence data are used (Robinson, 1954; Robinson and Stokes, 1965). A sea water of chlorinity 18‰, and salinity 32.52‰ is equivalent to 0.5263 molal NaCl with a concentration of 970 milliosmoles. At 20° C this sea water has a chloride concentration of 18.41 g Cl/liter.

Much of this work was done at the Millport Marine Station of the Scottish Marine Biological Association, before the move of the laboratory to its new site at Dunstaffnage, Oban.

RESULTS

Osmotic concentration of blood and muscle

Seven measurements of the osmotic concentration of plasma and of the juice expressed or centrifuged from muscle were made in *Squalus* (Table I). The plasma was hyperosmotic to sea water by 0.3–5.2% and in six out of the seven,

TABLE I

Osmotic concentration of blood and muscle of Squalus *(milliosmoles per kg water).*

No. and sex	Sea water	Blood plasma	Muscle juice	Plasma as % sea water	Muscle juice as % plasma
♂					
1	964	1005	997 (0.5 hr)	+104.3	99.2
2	968	989	1019 (0.5 hr)	+102.2	103.0
3	970	985	1006 (0.5 hr)	+101.5	102.1
4	968	971	1018 (1.2 hr)	+100.3	104.8
5	964	997	1013 (1.25 hr)	+103.4	101.6
6	958	1008	1087 (2.5 hr)	+105.2	107.8
Mean	965	993*	1023†	102.8	103.1
S.E.	±1.8	±5.6	±13.2	±0.75	±1.2
♀					
1	979	991	1030 (1.5 hr)	101.2	103.9
2	983	1004	—	102.1	—
3	983	1026	—	104.4	—
Mean	982	1007	—	102.6	—
S.E.	±1.4	±10.1	—	±0.95	—

Figures in parentheses are times between removal of muscle and completion of estimation. Muscle juice was obtained by tissue press from the 6 ♂♂, by means of the ultracentrifuge in the ♀.

* Significantly different from sea water by *t*-test ($P < 0.01$).

† Not significantly different from plasma ($P > 0.05$).

the muscle juice was hyperosmotic to the plasma. While the first difference is statistically significant (for the 6 ♂ specimens) the second is not, owing to the considerable variation. There is a tendency for the muscle juice to be more hyperosmotic the longer the completed time for preparation and estimation. Thus the 2.5 hr value of 107.8% that of the plasma is outside the mean + 2 S.D. of the remaining 5 values (106.2%) done within 1.25 hr. The higher values in the muscle juice are most probably due to some breakdown of labile constituents such as creatine phosphate.

Inorganic ions

Composition of plasma and whole muscle. Three comparisons of plasma, muscle and sea water showed fairly broad agreement, and the means are given in Table II. The sum of ions investigated in the plasma came to 51–59% of that in sea water. The six major ions of the plasma are lower than the corresponding ions in sea water, the mean magnesium and sulphate levels being 7–11% of those in the surrounding water. Sodium, chloride and potassium levels are relatively much higher at 52–75%, while calcium is only 30%.

TABLE II

Ionic composition of plasma and whole muscle in 3 ♂ specimens.

	mg-ions per kg water									Water (g per 1 or g per kg)
	Na	K	Ca	Mg	Cl	SO_4	Lactate	Total P*	Total	
Plasma	296	7.2	2.95	3.48	276	3.11	7.2	2.37	595†	959
S.D.	24.4	1.8	0.30	1.34	21.6	—	—	—	41	7
Muscle	42.4	119	2.09	12.9	35.9	1.24	23.8	91.3	329	761
S.D.	9.7	14.6	0.45	1.57	5.7	0.35	13.4	14.9	28	23
Sea water	453	9.6	9.9	51.6	529	27.3	—	—	1080	989

* mg-atoms.

† This total is slightly different from addition of mean plasma values (598), owing to absence of figures for SO_4, lactate and total P for one specimen.

In muscle the total concentration of ions is rather more than half that of the plasma. The increased potassium and magnesium of muscle, about 17 and 4 times plasma values, are offset by lower concentrations of sodium and chloride, which are about a seventh and an eighth of those in the plasma. The acid-soluble phosphorus of muscle, with a mean of 91 mg-atoms, is present as various phosphate compounds which act as anions; these are considered further below (see Table VIII). No special precautions were taken in the estimation of lactate, so that the values may be higher than *in vivo*. A lower plasma concentration of 2.9 mg-ions was found in a female specimen.

Apparent extracellular spaces in muscle and intracellular ionic concentrations. Intracellular concentrations of ions in muscle could be calculated if the extracellular space were known. The technique of incubating strips of muscle in a

TABLE III

Apparent extracellular spaces in muscle (concentrations as percentages of those in plasma, on water content basis) and calculated intracellular chloride (based on inulin injection experiments).

Specimen	Extracellular spaces as per cent total muscle water		Chloride mg-ions per kg water			Time allowed for inulin equilibration hr
	Inulin	Chloride	Plasma	Whole muscle	Muscle cells	
♂	13.22	13.84	309	42.8	2.3	15
♂	18.18	24.33	345	83.8	25.8	19
♂	4.31	13.90	283	39.4	28.4	22
♀	14.75	22.42	271	60.7	24.3	14
Mean	12.62	18.62	302	56.7	20.2	—
S.E.	±2.95	±2.77	±16.4	±10.2	±6.0	

Ringer solution containing inulin as used for determination of extracellular space in amphibian and rat muscle (Ling and Kromash, 1966) proved valueless for dogfish muscle, even with the animal's own plasma as medium. Chloride values in the muscle rose to 2–3 times their initial values after 6–11 hr at 10° C, although the inulin spaces remained at about a third of the chloride spaces.

Some estimates were obtained by injecting a solution of inulin in sea water into 4 animals, and measuring the inulin and chloride concentrations of plasma and muscle after 14–22 hr (Table III). The mean inulin space was about two-thirds that of the chloride space, indicating that some chloride is present inside cells, but the calculated intracellular values are rather variable, 2.3–28.4, with a mean of 20.2 mg-ions/kg cell water. Most of the whole muscle and plasma chloride concentrations of these specimens are higher than those in Table II, the mean muscle chloride space of the latter being 13.02%, compared with 18.62%. If the inulin space as measured was used to calculate the intracellular ionic concentrations of the specimens in Table II, there would be no chloride inside the cells. To get an approximate idea of average intracellular concentrations in these specimens it would seem appropriate to use the ratio inulin/chloride of 12.62/18.62 and apply it to the 13.02% chloride space. On this basis the extracellular space would be 8.82% and Table IV gives the calculated intracellular values. These are obtained from the

TABLE IV

Intracellular composition of muscle compared with plasma (calculated from data of Table II and an extracellular space of 8.82% muscle water.

	mg-ions per kg water							
	Na	K	Ca	Mg	Cl	SO_4	P*	Total
Muscle cells	17.9	130	2.01	13.8	12.7	1.06	100	277
Plasma	296	7.2	2.95	3.48	276	3.11	2.37	591
Ratio: $\frac{\text{muscle}}{\text{plasma}}$	0.060	18.1	0.68	4.0	0.046	0.34	42.2	0.47

* mg-atoms.

whole muscle analyses of Table II by subtracting values of plasma ions in 88.2 g of the total muscle water from the ionic concentrations of whole muscle, and recalculating the intracellular concentrations, now in 911.8 g water, to a kg water basis.

These intracellular concentrations show the normal pattern of high potassium and phosphate, and low sodium and chloride of muscle cells. Potassium and magnesium are the only cations to become concentrated in the cells, the former to eighteen times and the latter to four times the level in the plasma. Cellular sulphate and calcium remain low, calcium at about a seventh of the magnesium concentration. The ratios of K_i/K_o and Cl_o/Cl_i are respectively 18.1 and 21.7.

Muscle juice. The pale fawn juice centrifuged or pressed from muscle has an ionic composition similar to that of whole muscle when the analyses are given on a kg solvent water basis, the only marked differences being in their lower Ca (−43%) and Mg (−19.5%) values (Table V). Similarly the muscle juice and the piece of muscle from which it had been centrifuged or expressed differed only slightly except for marked reductions of Ca and Mg in the juice. The minor differences in the other ions are, however, significant at the 95% level, except those for K, and the Cl in the juice is higher in contrast to the lower values of the cations.

TABLE V

Composition of muscle juice, centrifuged or pressed muscle and whole muscle [mg-ions (mM) per kg solvent water].

Ion	Whole muscle	Muscle juice	Centrifuged or pressed muscle	Difference		Difference	
				Whole muscle muscle juice	*t*-test	Centrifuged muscle—muscle juice	*t*-test
Na	42.5 ± 9.1 (5)	40.1 ± 8.9 (5)	43.4 ± 9.3 (5)	2.40	3.27* (2.78)	3.36	3.24* (2.78)
K	128 ± 8.0 (6)	124 ± 7.5 (6)	132 ± 8.9 (6)	3.7	2.24 (2.57)	7.7	2.33 (2.57)
Ca	2.60 ± 0.56 (5)	1.49 ± 0.38 (5)	3.06 ± 0.64 (5)	1.11	5.25* (2.78)	1.58	5.31* (2.78)
Mg	11.81 ± 0.94 (5)	9.51 ± 0.76 (5)	13.41 ± 0.90 (5)	2.31	4.00* (2.78)	3.90	8.63* (2.78)
Cl	41.9 ± 7.6 (6)	44.9 ± 7.5 (6)	40.4 ± 7.9 (6)	−2.98	2.71* (2.57)	−4.43	3.72* (2.57)

* The mean difference between the paired observations is significant at $P = 0.05$, calculated t exceeding the value from table t given in brackets.

Mean water contents mg per kg are: original muscle 748 ± 6.4 (9), muscle juice 885 ± 2.4 (20), centrifuged muscle 699 ± 5.2 (20). ± = S.E. ( ) = N.

The purpose of these analyses was to demonstrate any ion binding which would be apparent by reductions in the ions of muscle juice compared with those in whole muscle and in centrifuged muscle. Extracellular fluid in muscle and muscle juice complicates any calculations of binding since it may form a different proportion of the solvent water in the two cases. In only one specimen was the inulin space determined in both muscle and muscle juice, the ratios found being 1.00 : 1.16. Using 10% and 11.6% extracellular volumes in the water of whole muscle and muscle juice, respectively, one can calculate the composition of muscle cells and that of the cellular component of the juice (Table VI). This calculation suggests that about half of the cellular Na and Ca and a fifth of the Mg are held back when muscle is centrifuged, perhaps bound in complexes by the structural proteins, actin and myosin, and possibly by the sarcolemma. None of the K would appear to be bound, and only 8% of the Cl. It is improbable that even this amount of Cl is

TABLE VI

Approximate amount of ion-binding (based on data of Table V), using extracellular volume of 10% and 11.6% in muscle and muscle juice, respectively.

	mg ions (mM) per kg water				
	Na	K	Ca	Mg	Cl
Muscle cell	14.3	141	2.56	12.7	15.9
Cellular portion of muscle juice	6.5	139	1.30	10.3	14.6
Bound ions (%)	55	1.4	49	19	8

bound. A slightly smaller extracellular volume in the muscle juice (11.2%) would result in negligible Cl binding (0.6%), while still leaving the same proportions of Ca and Mg bound, slightly less Na (45%) bound, and still only 1% of the K.

Organic constituents of plasma and muscle

Nitrogenous constituents. In Table VII are set out analyses of 7 nitrogenous constituents (excluding protein) in plasma and muscle: urea, trimethylamine oxide, betaine, free amino acids, creatine, creatinine and ammonium ions. On the average about 87.4% of the NPN of plasma, and 87.5% of the NPN of muscle are accounted for by these constituents, and if are added the ATP-N (see Table VIII) and amide-N (latter 3.27 and 5.72 mM in plasma and muscle respectively), about 92% of the NPN of muscle is attributable to known compounds and ions. While concentrations of urea in plasma and muscle are high but very similar, those of muscle TMAO are 3 times as high as plasma values. Because of these high values in muscle and the large amounts of the nitrogenous bases creatine and

TABLE VII

Nitrogenous constituents of plasma ana muscle.

	mM per kg water							
	Urea	TMAO	Betaine	Creatine	Creatinine	Amino-N	NH_4	Total NPN*
Plasma	308	72.4	9.1	0.126	0.046	11.6	0.40	838
S.D.	31.3	15.0	13.5	0.09	0.009	3.9	0.46	93.3
N	7	7	4	3	3	4	3	4
Muscle	333	180	100	68.2	1.02	108	4.7	1447
S.D.	18.8	31.6	33.5	8.3	0.81	24.0	3.8	89.2
N	7	7	7	13	3	7	4	7

* mg-atoms.
TMAO = trimethylamine oxide.
NPN = non-protein nitrogen.

betaine, together with the 10-times larger concentration of amino acids, muscle NPN is greater than plasma NPN by a factor of about 1.7.

Creatine is present in muscle partly as creatine phosphate, and this bound creatine was found in 3 estimations to be about a half of the total creatine (25.6, S.D. 6.2, compared with 62.9, S.D. 5.4 mM per kg water).

The phosphorus compounds. Phosphate compounds in ice-cold trichloroacetic acid extracts of muscle were separated into four fractions, inorganic phosphate, adenosine triphosphate (ATP), creatine phosphate and a fourth fraction, containing hexose phosphate *etc.*, by subtracting the sum of the first three from the total acid-soluble P. In the 3 specimens examined the labile creatine phosphate forms about 28% of the total P, inorganic phosphate 33%, ATP 11% and the remaining fraction 27% (Table VIII).

TABLE VIII

Acid-soluble phosphate fractions in muscle.

mg-ions per kg water				
Inorganic phosphate	Creatine phosphate	Adenosine triphosphate	Hexose phosphates, etc	Total phosphate
28.0 S.D. 5.9 (3)	23.9 6.2 (3)	9.6 0.79 (3)	22.9 5.5 (3)	84.4 5.9 (3)

Creatine phosphate as measured here from phosphate fractionation should be of the same order as the bound creatine determined by the α-naphthol diacetyl method. Values using the latter method for the same specimens showed broad agreement, 32, 26 and 19 mM per kg water as against 27, 28 and 17 mM as creatine phosphate.

Minor organic and inorganic constituents. Glucose and glycerol contribute little to the osmotic concentration of muscle. Glycerol values in muscle were below 1 mM [0.77, S.D. 0.22 (3)]. Of 5 specimens in which both the plasma and muscle glucose were determined, the values were within 4% of each other only in two cases. Means were 6.29, S.D. 3.76 (7) in plasma, and 5.06, S.D. 2.61 (8) in muscle. Total CO_2 in muscle was 7.08, S.D. 0.40 (5), the mean HCO_3 component being 6.81 at pH 7.5, and plasma inorganic P was 1.58, S.D. 0.88 (5).

Osmolality of plasma and muscle

From a summation of the analyzed ions and organic constituents we can see how far the totals of plasma and sea water, and of plasma and muscle, agree with each other (Table IX), and these totals can be compared with the measured osmolality of other specimens. The data are from three male specimens in which ions, nitrogenous compounds, glucose and glycerol were studied in both plasma and muscle. Neglecting for the moment the osmotic coefficients of ions and molecules, and the possibility that some of the ions are bound, it is seen that the plasma total of ions and molecules of 1116 is only 3% higher than the sea water ions. Inorganic and organic ions, urea and trimethylamine oxide form 89% of the total. Compared with plasma the total concentration of muscle is 7.7% higher. A reduc-

TABLE IX

Osmolality of plasma, whole muscle and sea water (mean of 3♂ specimens).

Constituent	mg-ions + mM per kg water			Osmotic coefficient	milliosmoles per kg water		
	Plasma	Muscle	Sea water		Plasma	Muscle	Sea water
Inorganic ions, phosphate *etc.*	606	291	1083	0.90	545	262	975
Urea	314	332	—	0.96	301	319	—
Trimethylamine oxide	76	172	—	1.19	90	205	—
Betaine	—	101	—	1.115	—	113	—
Amino acids and amides	13	118	—	?1.0	13	118	—
Creatine and Creatinine	0.2	61	—	?1.0	0.2	61	—
NPN unaccounted for	101*	121*	—	?1.0	?101	?121	—
Glucose and Glycerol	6	6	—	1.0	6	6	—
Total	1116	1202	1083		1056	1205	975

Creatine phosphate (23.6 mg-ions) subtracted from the ionic phosphate values of muscle since it is included in the total creatine.

* mg-atoms.

tion of ions to half those of the plasma is counterbalanced by more than a doubling of TMAO and a large increase in free amino acids and creatine, as well as the presence of 101 mM betaine.

It is possible that the unknown NPN of plasma and muscle may include compounds with more than one nitrogen atom per molecule, which would reduce both total concentrations. The dipeptides anserine and carnosine found in teleost muscle seem to be absent in dogfish muscle filtrates (unpublished work of C. B. Cowey and J. D. Robertson).

To convert the data to milliosmoles requires the use of osmotic coefficients which may depart from unity in both salts (ions) and organic compounds. These are given in the fifth column of Table IX. It is assumed that plasma and muscle ions have approximately the same osmotic coefficient as sea water ions. For a chlorinity of 18.00‰ (18.61 g Cl per kg solvent water), this can be calculated from the osmotic equivalence data of Robinson (1954) and the g-ionic concentration of sea water [1.1368 at Cl 19.00‰ as given by Sverdrup, Johnson and Fleming (1942)]. The value at Cl 18.00‰ is 969.9/1074.0 = 0.903, this being the osmotic equivalence in milliosmoles divided by the total mg-ions (mM) by analysis. The urea coefficient is from Robinson (1954), betaine from Smith and Smith (1940) and trimethylamine oxide by own determination as no value was found in the literature. Like the other dipolar compound betaine the value obtained for trimethylamine oxide exceeded unity substantially, being 1.19. It was determined on a molal solution of trimethylamine oxide dihydrate (Eastman chemical) by the vapour pressure method given in the introduction, using for comparison an organic standard of molal DL-alanine, which is an almost ideal solute with an osmotic coefficient of 1.003 (Robinson, 1952). Correction was made for the fact that an assay of the TMAO solution showed the compound to be 99.0% pure, as determined from its N (micro-Kjeldahl) and trimethylamine components (99.3% and 98.7%, respectively, of the theoretical).

A value of 1.0 has been taken for the average osmotic coefficient of the amino acids. At 1 molal the coefficient is 0.928 for glycine (Smith and Smith, 1937) and 1.046 for proline (Smith and Smith, 1940), but apart from alanine just mentioned, no published values for the amino acids have been found. In *Squalus* muscle the most abundant of the free amino acids are proline, glycine, taurine, alanine and sarcosine (C. B. Cowey and J. D. Robertson, unpublished). Proline, glycine and alanine formed on the average 68% of the total amino acids (104 mM per kg water) in 3 specimens, and using the coefficients 70.3 millimoles becomes 70.6 milliosmoles.

After conversion of all the data to milliosmoles the total concentration of plasma comes to 1056, 8.3% higher than the sea water in which the specimens were living (*cf.* Table I, where six male specimens of *Squalus* were hyperosmotic by 2.8%). The muscle concentration at 1205 milliosmoles is 14.1% higher than that of the plasma. Regarding the unknown non-protein nitrogen of the plasma a small fraction may be betaine (Table VII). Some of the remainder may be in peptide form, or even be present as a trace of non-precipitated protein; either or both of these possibilities would reduce the NPN unaccounted for and so also the calculated osmolality of the plasma. To get a calculated osmolality of plasma similar to that found by direct vapour pressure measurement of other specimens would require some 54 of the 101 mg-atoms N to be present in compounds of negligible osmotic concentration, that is, of high molecular weight.

The same suggestion may be put forward for the apparently excessive calculated osmotic concentration of muscle; some of the 121 mg-atoms NPN may be present in macromolecular form. A slight correction can, however, be made to it from knowledge of the free amino acids. The calculation of unaccounted for NPN was made on the assumption that the 112 mg-atoms α-amino N atoms found in these 3 specimens were from amino acids with a single N. In fact, from unpublished work the presence of lysine (2 N-atoms), histidine (3) and arginine (4) means that the 112 mM amino acids contain 121 mg-atoms N. The total of 121 mg-atoms NPN unaccounted for then becomes 112, and total milliosmoles for the muscle 1196.

The last factor to be considered is binding of ions. In plasma this is probably negligible, concerning a fraction of the 2.95 mg-ions calcium. In muscle binding it concerns inorganic ions and possibly some of the organic phosphate ions. On a basis of the binding of 45% Na, 1.4% K, 49% Ca, 19% Mg and 0.6% Cl of the intracellular ions (Table VI and text), a muscle value of 291 mg-ions (Table IX, based on Tables II and VIII) and an extracellular space of 8.82% of muscle water, the concentration of these ions in whole muscle, 212 mg-ions, is reduced by 12 mg-ions or 11 milliosmoles. Thus quantitatively the binding of these ions in *Squalus* muscle is not very important. The total ions are now (262–11) = 251 milliosmoles, bringing the total osmotic concentration from 1196 (corrected from 1205) to 1185. This total is still 12% higher than that calculated for the muscle, which may be compared with the 3.1% difference found between muscle juice and plasma (Table I).

As a general check on the validity of converting the analytical data to milliosmoles, using osmotic coefficients, a solution closely resembling *Squalus* plasma was made up, containing salts, urea and TMAO. Its composition in mM per kg water was NaCl 296, KCl 7.2, $CaCl_2$ 2.9, $MgCl_2$ 3.5, Na_2SO_4 5.7, $NaHCO_3$ 6.8, urea 289 and TMAO 71. The total molality of the salts came to 656.3 which, with an

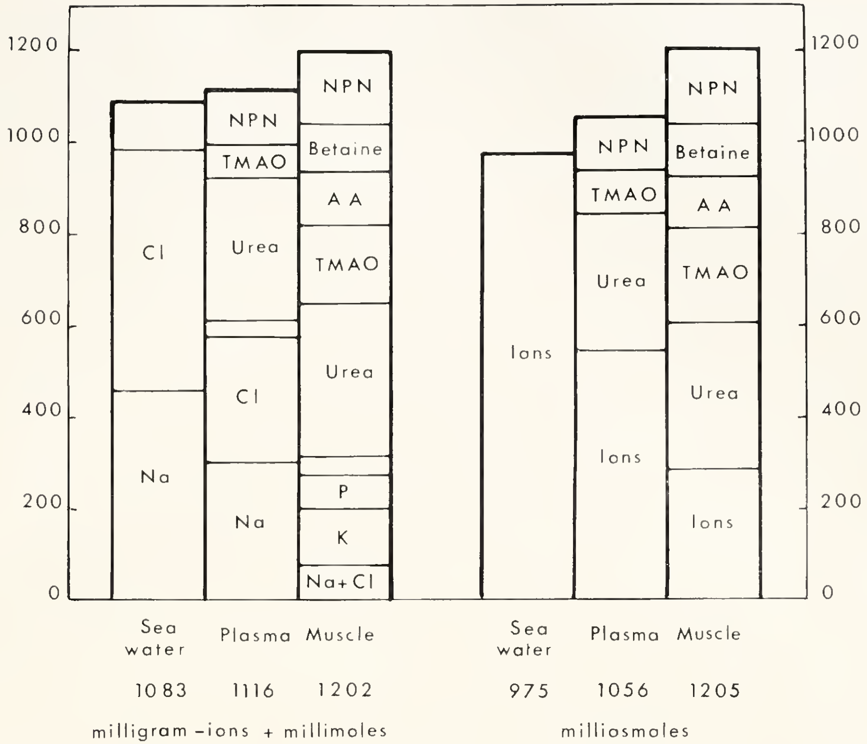


FIGURE 1. Composition of plasma and muscle of *Squalus acanthias* in relation to sea water; AA—free amino acids; TMAO—trimethylamine oxide; NPN—remaining non-protein nitrogen (in mg-atoms). The unlabeled sections in the columns are the other ions of plasma and muscle. Scale on left—ions and molecules as analyzed, scale on right—milliosmoles as calculated using osmotic coefficients.

osmotic coefficient of 0.903, is equivalent to 592.6 milliosmoles. Urea of 289 mM gives 277.4 milliosmoles (coefficient of 0.96) and TMAO of 71 mM equals 84.5 milliosmoles (coefficient of 1.19). Thus the estimated molality is 954.9 milliosmoles for an artificial plasma (minus protein) containing 1016.3 millimoles of salts and nitrogenous compounds. The osmolality, using the Krogh-Baldes vapor pressure thermoelectric method and NaCl standards, was found to be 948 milliosmoles, a difference of only 0.7%.

Figure 1 shows the principal constituents of plasma and muscle. The balance of NPN unaccounted for has been left uncorrected and is in mg-atoms. The chief features of the muscle compared to plasma are (1) the great reduction in muscle ions, the increase in K being far outweighed by decreases in Na and Cl, and (2) the great increase in TMAO, amino acids and betaine.

DISCUSSION

It seems well established that the blood serum or plasma of elasmobranchs is slightly hyperosmotic to sea water (Holmes and Donaldson, 1969). In the spiny dogfish the writer's values, about 3% higher than the medium, are rather lower than most of the previous data for this species. Certainly the concentration of blood plasma in the *Squalus* from the Clyde sea-area shows a smaller mean difference from that for specimens of the same species from Salisbury Cove, Maine, where Burger (1967) gives a further value slightly below that of Burger and Hess (1960) of 1000 milliosmoles compared with 930 in sea water (+ 7.5%).

Comparison of the major ions in Table II with fairly complete analyses of the blood plasma or serum of *Squalus* from the eastern coasts of the United States and Canada shows good agreement. The values of Rodnan, Robin and Andrus (1962) and those of Macallum (1910), the latter obtained by classical gravimetric methods, in brackets, are Na 263 (296), K 4.1 (7.4), Ca 3.3 (4.3), Mg 1.55 (6.4), and Cl 249 (295). Macallum's data have been recalculated to mg-ions per kg water, those of Rodnan *et al.* (1962) left as mg-ions per liter. A 4% increase to get the data of Rodnan *et al.* in terms of weight of solvent water would bring the analyses even nearer to each other. The chief differences are in K and Mg.

Potassium values in plasma or serum are particularly prone to be higher than normal if the slightest tinge of hemoglobin indicates some slight hemolysis and leakage of K from the red blood cells. Cserr and Rall (1967) claim that the lowest values in the spiny dogfish are obtained if sampling from the caudal vein is immediate. Normal values of K 4.1 ± 0.12 mg-ions per liter rise 50% if the sampling is delayed 7–15 minutes after the fish is taken from the water. Despite complete lack of obvious hemolysis, the mean value of K 7.2 in Table II may be rather high. A further 2 ♂♂ gave 5.8 and 6.4, while 3 ♀♀ gave a mean of 4.8 (4.11, 4.38, 5.84), these values being in mg-ions (m. equiv.) per liter.

Lactate is an ion which shows considerable variation. The values of 6.0 and 8.3 mg-ions in 2 ♂♂ (mean 7.2 of Table II) and the lower figure of 2.9 in a ♀ may be compared with data given by Murdaugh and Robin (1967), in which elevated values in the laboratory specimens (sex unstated of 7.9 ± 5 (18) mg-ions per liter contrasted with lower values of 4.5 (10) in those kept in live cars in the dock area, and still lower, 0.94 ± 0.3 (11) in freshly captured specimens sampled within a minute.

Total inorganic ions of plasma in *Squalus* come to only 52% of the osmolarity in the analyses of Rodnan *et al.* (1962) and to 54% of the osmolality (based on concentrations per kg solvent water) in the present work (Table IX). Another value based on freezing points of original serum and ashed serum made up to its original volume is 53% (Macallum, 1910). Nitrogenous compounds, particularly urea, trimethylamine oxide, and to a minor extent free amino acids and perhaps betaine, are responsible for most of the balance, since glucose is relatively insignificant.

Urea values in *Squalus*, 288–350, mean 308 ± 31.3 (± = S.D.) mM per kg water (Table VII) may be compared with 357 ± 32 mM per liter based on an unstated number of plasma analyses by Rodnan *et al.* (1962), and 4 values, range 347–352 mM per liter of Burger and Hess (1960). Trimethylamine oxide at 72.4 ± 15, range 54–95 mM per kg water (Table VII) is in complete agreement with the data of Cohen, Krupp and Chidsey III (1958), 71 ± 10 (N = 39) mM per liter. A single value of 64 mM has been given for the serum of the North Pacific form of *Squalus acanthias* ('*S. suckleyi*') by Norris and Benoit (1945).

The only comprehensive data on dogfish plasma with which the present work on *Squalus* may be compared are those of Doolittle, Thomas and Stone (1960) on the smooth dogfish *Mustelus canis*. In a total of 1037 mM per kg water, ions account for 56%, urea 33% and TMAO 9.4%. The two nitrogenous compounds are rather higher than in *Squalus*, mean urea being 342 mM and TMAO 97 mM, but the individual ions are very similar. If the osmotic coefficients in Table IX are applied to the data, ions come to 527, urea to 328 and TMAO to 115 milliosmoles which together with glucose add up to 983. This may be compared with their directly estimated 962 and 910 milliosmoles (from freezing-point depression) for *Mustelus* plasma and the sea water in the tank at Woods Hole. Agreement is good, but total NPN was not estimated.

A study on the electrolytes of muscle in *Squalus* has been made by Robin, Murdaugh and Weiss (1964) who analyzed 10 fish for Na, K and Cl. They calculated intracellular concentrations on the basis of exclusion of Cl from muscle cells, using the chloride space (muscle Cl as % plasma Cl, both on a water content basis) as a measure of extracellular space. The chloride spaces found were 14 ± 2% (range 11–17), which may be compared with those from the data of Tables II and III of 13.0 and 18.6% (means for 3 and 4 dogfish respectively). Plasma Na and K (means) were 240 and 3.6, with whole muscle means of 55.4 (38–75) and 162 (104–216) mg-ions per kg water. Thus while Na values are comparable with data in Tables II and V, most of the muscle K concentrations of Robin *et al.* (1964) exceed those in this paper, mean 125 (9), range 95–146. Calculated intracellular concentrations are higher for K, 187 ± 42, compared to 130 and 141 in the present work, and higher also for Na, 29.5 ± 9, as against 17.9 and 14.3 (Tables IV and VI). If Cl is present inside muscle cells, as is probable from a comparison of chloride with inulin space (Table III), the calculated intracellular concentrations in the American specimens would be altered, resulting in a lower K and a higher Na. However, some variation in ionic concentrations must be expected and the two sets of data are broadly consistent. Some variation in extracellular and intracellular ions must arise from a degree of flexibility in the proportion of ions and nitrogeneous molecules making up the osmotic concentration. An exceptionally low urea and NPN in the plasma of one specimen was offset by a higher Cl, indicating some such mutual adjustment.

Juice expressed from muscle has an osmotic concentration averaging 3% higher than that of plasma (Table I). It would seem that this reflects a slight breakdown of labile constituents during the pressing or centrifugation of the muscle, so that *in vivo* muscle is probably almost isosmotic with the plasma.

While the concentrations of K and of both inorganic and organic phosphate are high in muscle, the Na and Cl are so low that the proportion of the osmotic concentration due to ions is only about a half what it is in plasma. Nitrogenous compounds make up the balance. Urea is only silghtly higher than in plasma, in the specimens analyzed 333 and 308 mM per kg water, but there are large increases in creatine, mean 68.2 mM from less than 1 mM in the plasma, TMAO 180 mM as against 72 mM, betaine 100 mM from very small amounts, and free amino acids 108 mM compared to 12 mM (Table VII).

Data comparing blood and muscle concentrations of these compounds in *Squalus* seem to exist only for TMAO. Goldstein, Hartman and Forster (1967) give

3 comparisons in which muscle concentrations (per kg water) were 3 times those of the plasma, 225, 154 and 216 mM compared to 76, 45 and 78 mM, respectively. These values are all within the ranges given in Table VII.

Extensive data on the nitrogenous extractives of *Squalus acanthias* muscle have been given by Vyncke (1970). Recalculation of his mean data and ranges for nitrogenous compounds on a millimolal basis gives urea 415 (351–480), TMAO 189 (165–212), betaine 11.0 (3.3–31.7), creatine 51.1 (26.5–65.7), creatinine 6.2 (2.0–10), ammonium 18.4 (5.6–33.7), α-amino N 61 ± 13 in mature, 74.2 ± 14 in immature dogfish. The analyses in Table VII are in general agreement with these means and ranges, except that amino acids and betaine are much higher, and urea, creatinine and ammonium ions are in the lower ranges. In the present work creatinine and ammonium ions were determined immediately, since their values rise in extracts kept in the refrigerator. In trichloroacetic acid solution creatine slowly changes to a mixture of creatinine and creatine, the values of creatinine rising as creatine falls.

The question of betaine must be considered. In muscle of three elasmobranchs including *Squalus*, Shewan (1953) found that TMAO and betaine were the two largest of the nitrogenous extractives, but no absolute values for betaine were given. He later (Shewan, 1961) gave a figure of 1500 mg per kg muscle. Assuming a water content of 75%, this amounts to only 17 mM per kg water. Vyncke's (1970) maximum value is only 32 mM. Much time was spent on getting a fairly satisfactory method of analysis and the writer considers his value of 100 (52–145) mM to be valid. This amount is comparable to that found in decapod crustacean and cephalopod muscles (*e.g.*, 99 mM in *Sepia* whole muscle, Robertson, 1965). *Squalus* plasma seems to have a low betaine concentration, < 4 mM. An exceptional 29.2 mM was found in a dogfish with a muscle value of 121 mM but other plasma values were 0.4, 3.0 and 3.7 (mean 9.1, Table VII).

The principal aim of this work was to get a comprehensive analysis of muscle and plasma and to see how far the totals of the principal osmotic constituents agreed with each other and with direct measurements of plasma and muscle osmotic concentration. On the basis of chemical estimations, the calculated osmolality of known ions and compounds (means of 3 ♂♂ dogfish) is 955 in plasma compared with 975 milliosmoles in sea water. But a further 101 mg-atoms of plasma NPN has to be added, which, if present in compounds with 1 N atom per molecule, would give a calculated value 8.3% higher than the sea water. Possibly some of the nitrogen is in peptide form, which would reduce the difference. The actual mean difference in 6 ♂♂ was + 3% (Table I).

For muscle the calculated osmolality of known ions and molecules is 1084 milliosmoles, but it has a further 121 mg-atoms of N unaccounted for. Some of this is probably peptide-N, as Vyncke (1970 finds 59 ± 13 mg-atoms in *Squalus* muscle. Disregarding it for the moment, the known ions and compounds (1084) come to a value 2.7% higher than that of the plasma, in good, perhaps fortuitous, agreement with the measured + 3% in other specimens (Table I). Including it, the total is 1205, + 8.3% on the plasma value. Presumably this higher value would be reduced (1) if any breakdown of labile compounds from the *in vivo* condition could be allowed for, (2) if some of the N is in petpide form, and (3) if a proportion of any of the ions and molecules is bound in complexes with the cellular proteins. Cohen *et al.* (1958) have shown by dialysis that no binding of TMAO occurs in the plasma of *Squalus*. It is possible that some binding of nitrogenous constituents does occur in muscle, such as part or all of the ATP. Although it is inferred that some of the cellular Na, Ca and Mg is bound (Table VI), the concentrations of these ions are so small that the reduction due to binding (minus 11 milliosmoles) causes only a minor alteration to the osmolality of whole muscle.

A brief comparison may be made between dogfish and members of two other groups, *Myxine glutinosa*, a marine cyclostome isosmotic with sea water, and *Latimeria chalumnae*, the marine crossopterygian. According to Griffith, Umminger, Grant, Pang and Pickford (1974) ions in the blood serum of *Latimeria* come to 435.4 mg-ions per liter, and with urea 377 mM, TMAO 122 mM, amino acids 15.6 mM and glucose 6.6 mM, the total is 957. Using the osmotic coefficients in Table IX this total equals 922 milliosmoles, in complete agreement with the directly measured osmolarity of 932 milliosmoles per liter, sea water being 1035. No excess of NPN was found, the N of the known compounds exceeding the total NPN (856 mg-atoms) by 4%. The nitrogenous compounds of *Latimeria* muscle from a frozen specimen included 422 mM urea and 290 mM TMAO (Lutz and Robertson, 1971). Thus these constituents in both blood and muscle are higher in *Latimeria* than in *Squalus*. Contrasting with both of these fishes is the cyclostome *Myxine* in which nitrogenous constituents are practically absent from the plasma, ions forming about 99% of the osmotic concentration (Robertson, 1966). *Myxine* muscle is also different from that of these fishes, in that urea is practically absent (1.5 mM). It does contain TMAO 87 mM and betaine 65 mM per kg water, concentrations which are much lower than in *Squalus*. It makes up for this by having a high concentration of free amino acids, 291 mM, so that the total nitrogenous consituents form about 55% of the osmotic concentration in *Myxine*, compared to about 69% in *Squalus*.

I am indebted to the Director of the Dunstaffnage Marine Research Laboratory, Mr. R. I. Currie, for facilities at Millport. I wish to thank those of his staff, particularly Mr. E. Latham and Mr. W. S. Finlayson, who provided *Squalus* in such good condition. Dr. J. A. Colin Nicol kindly read the proofs of this paper.

SUMMARY

Comprehensive analyses of the osmotic consituents of plasma and muscle of *Squalus acanthias* have been made. In plasma mean concentrations of ions and molecules (mg-ions or mM per kg water) were Na 296. K 7.2, Ca 2.95, Mg 3.48, NH_4 0.4, Cl 276, SO_4 3.11, HCO_3 6.8, lactate 7.2, urea 308, trimethylamine oxide (TMAO) 72.4, amino-N 11.6, total NPN 838 mg-atoms. In muscle mean concentrations were Na 42.4, K 119, Ca 2.09, Mg 12.9 Cl 35.9, SO_4 1.24, lactate 23.8, total P (mg-atoms) 91.3, urea 333, TMAO 180, betaine 100, creatine 68.2 (26% bound as creatine phosphate), amino-N 108, NH_4 4.7, total NPN 1447 mg-atoms. In other specimens fractionation of the acid-soluble compounds in muscle gave inorganic P 28.0, creatine phosphate 23.9, ATP 9.6, remaining phosphate 22.9 (total 84.4 mg-ions).

Using osmotic coefficients calculated osmotic concentration of muscle exceeded that of plasma, and possible reasons for this are discussed. Directly determined

osmotic concentrations were sea water 965, plasma (6 ♂♂) 993, and muscle juice 1023 milliosmoles.

Some estimates of intracellular ionic concentrations were obtained from measurements of inulin space in muscle. Analysis of muscle juice enabled approximate estimates of ion-binding. About half the Na and Ca and nearly one fifth of the Mg in muscle cells appears to be bound, but little of the Cl and K.

LITERATURE CITED

BURGER, J. W., 1967. Problems in the electrolyte economy of the spiny dogfish, *Squalus acanthias.* Pages 177–185 in P. W. Gilbert, R. F. Mathewson and D. P. Ryall, Eds., *Sharks, Skates and Rays.* The John Hopkins Press, Baltimore, Maryland.

BURGER, J. W., AND W. N. HESS, 1960. Function of the rectal gland in the spiny dogfish. *Science*, **131**: 670–671.

COHEN, J. J., M. A. KRUPP AND C. A. CHIDSEY III, 1958. Renal conservation of trimethylamine oxide in the spiny dogfish, *Squalus acanthias. Amer. J. Physiol.*, **194**: 229–235.

CONWAY, E. J., 1962. *Microdiffusion Analysis and Volumetric Error.* (5th ed.) Crosby Lockwood, London, 467 pp.

CSERR, H., AND D. P. RALL, 1967. Regulation of cerebrospinal fluid (K^+) in the spiny dogfish, *Squalus acanthias. Comp. Biochem. Physiol.*, **21**: 431–434.

DOOLITTLE, R. F., C. THOMAS AND W. STONE, JR., 1960. Osmotic pressure and aqueous humor formation in dogfish. *Science*, **132**: 36–37.

DYER, W. T., 1952. Amines in fish muscle. VI. Trimethylamine oxide content of fish and marine invertebrates. *J. Fish. Res. Board, Can.*, **8**: 314–324.

ENNOR, A. H., 1957. Determination and preparation of N-phosphates of biological origin. I. Phosphocreatine. Pages 850–856 in S. P. Colowick and N. K. Kaplan, Eds., *Methods in Enzymology*, Vol. 3. Academic Press, New York.

FRAME, E. G., J. A. RUSSELL AND A. E. WILHELMI, 1943. The colorimetric estimation of amino nitrogen in blood. *J. Biol. Chem.*, **149**: 255–270.

GOLDSTEIN, L., S. C. HARTMAN AND R. P. FORSTER, 1967. On the origin of trimethylamine oxide in the spiny dogfish, *Squalus acanthias. Comp. Biochem. Physiol.*, **21**: 719–722.

GRIFFITH, R. W., B. L. UMMINGER, B. F. GRANT, P. K. T. PANG AND G. E. PICKFORD, 1974. Serum composition of the coelacanth, *Latimeria chalumnae* Smith. *J. Exp. Zool.*, **187**: 87–102.

HEAGY, F. C., 1948. The use of polyvinyl alcohol in the colorimetric determination of magnesium in plasma or serum by means of titan yellow. *Can. J. Res. Sec. E*, **26**: 295–298.

HOLMES, W. N., AND E. M. DONALDSON, 1969. The body compartments and the distribution of electrolytes. Pages 11–89 in W. S. Hoar and D. J. Randall, Eds., *Fish Physiology*, Vol. 1. Academic Press, New York.

HULLIN, R. P., AND R. L. NOBLE, 1953. The determination of lactic acid in microgram quantities. *Biochem. J.*, **55**: 289–291.

KERMACK, W. O., H. LEES AND J. D. WOOD, 1955. Some non-protein constituents of the tissues of the lobster. *Biochem. J.*, **60**: 424–428.

KROGH, A., 1933. Extracellular and intracellular fluid. *Acta Med. Scand. Suppl.*, **90**: 9–18.

KROGH, A., 1939. *Osmotic Regulation in Aquatic Animals.* University Press, Cambridge, 242 pp.

LAMBERT, M., AND A. C. NEISH, 1950. Rapid method for estimation of glycerol in fermentation solutions. *Can. J. Res. Sec. B*, **28**: 83–89.

LING, G. N., AND M. H. KROMASH, 1966. The extracellular space of voluntary muscle. *J. Gen. Physiol.*, **50**: 677–694.

LUTZ, P. L., AND J. D. ROBERTSON, 1971. Osmotic constituents of the coelacanth *Latimeria chalumnae* Smith. *Biol. Bull.*, **141**: 553–560.

MACALLUM, A. B., 1910. The inorganic composition of the blood in vertebrates and invertebrates, and its origin. *Proc. Roy. Soc. B*, **82**: 602–624.

MURDAUGH, H. V., AND E. D. ROBIN, 1967. Acid-base metabolism in the dogfish shark. Pages 249–270 in P. W. Gilbert, R. F. Mathewson and D. P. Rall, Eds., *Sharks, Skates and Rays.* The Johns Hopkins Press, Baltimore, Maryland.

NORRIS, E. R., AND G. J. BENOIT, JR., 1945. Studies on trimethylamine oxide. I. Occurrence of trimethylamine oxide in marine organisms. *J. Biol. Chem.*, **158**: 433–438.

OWEN, J. A., B. IGGO, F. J. SCANDRETT AND C. P. STEWART, 1954. The determination of creatinine in plasma and serum, and in urine; a critical examination. *Biochem. J.*, **58**: 426–437.

ROBERTSON, J. D., 1949. Ionic regulation in some marine invertebrates. *J. Exp. Biol.*, **26**: 182–200.

ROBERTSON, J. D., 1960. Studies on the chemical composition of muscle tissue. I. The muscles of the hagfish *Myxine glutinosa* L. and the Roman eel *Muraena helena* L. *J. Exp. Biol.*, **37**: 879–888.

ROBERTSON, J. D., 1965. Studies on the chemical composition of muscle tissue. III. The mantle muscle of cephalopods. *J. Exp. Biol.*, **42**: 153–175.

ROBERTSON, J. D., 1966. Osmotic constituents of the blood plasma and parietal muscle of *Myxine glutinosa* L. Pages 631–644 in H. Barnes, Ed., *Some Contemporary Studies in Marine Science*. Allen and Unwin, London.

ROBIN, E. D., H. V. MURDAUGH, JR., AND E. WEISS, 1964. Acid-base, fluid and electrolyte metabolism in the elasmobranch. I. Ionic composition of erythrocytes, muscle and brain. *J. Cell. Comp. Physiol.* **64**: 409–418.

ROBINSON, R. A., 1952. The vapor pressure of aqueous solutions of alanine. *J. Biol. Chem.*, **199**: 71–73.

ROBINSON, R. A., 1954. The vapor pressure and osmotic equivalence of sea water. *J. Mar. Biol. Ass. U.K.*, **33**: 449–455.

ROBINSON, R. A., AND R. H. STOKES, 1965. *Electrolyte Solutions.* (2nd ed. revised) Butterworths, London.

RODNAN, G. P., E. D. ROBIN AND M. H. ANDRUS, 1962. Dogfish coelomic fluid: I. Chemical anatomy. *Bull. Mt. Desert Island Biol. Lab.*, **4**: 69–70.

ROE, J. H., J. H. EPSTEIN AND N. P. GOLDSTEIN, 1949. A photometric method for the determination of inulin in plasma and urine. *J. Biol. Chem.*, **178**: 839–845.

RUSSELL, J. A., 1945. Note on the colorimetric determination of amino nitrogen. *J. Biol. Chem.*, **156**: 467–488.

SHEWAN, J., 1953. The nitrogenous extractives from fresh fish muscle. II. Comparison of several gadoid and elasmobranch species. *J. Sci. Food Agric.*, **4**: 565–568.

SHEWAN, J. M., 1961. The microbiology of sea-water fish. Pages 487–560 in G. Borgstrom, Ed., *Fish as Food*, Vol. 2. Academic Press, New York.

SMITH, H. W., 1929. The composition of the body fluids of elasmobranchs. *J. Biol. Chem.*, **81**: 407–419.

SMITH, P. K., AND E. R. B. SMITH, 1940. Thermodynamic properties of solutions of amino acids and related substances. V. The activities of some hydroxy- and N-methylamino acids and proline in aqueous solution at twenty-five degrees. *J. Biol. Chem.*, **132**: 57–64.

SMITH, R. B., AND P. K. SMITH, 1937. The activity of glycine in aqueous solution at twenty-five degrees. *J. Biol. Chem.*, **117**: 209–216.

SUMNER, J. B., 1944. A method for the colorimetric determination of phosphorus. *Science*, **100**: 413–414.

SVERDRUP, H. V., M. W. JOHNSON AND R. A. FLEMING, 1942. *The Oceans.* Prentice Hall, New York, 1087 pp.

UMBREIT, W. W., R. H. BURRIS AND J. F. STAUFFER, 1949. *Manometric Techniques in Tissue Metabolism.* (2nd ed.) Burgess, Minneapolis, 227 pp.

VYNCKE, W., 1970. Influence of biological and environmental factors on nitrogenous extractives of the spurdog *Squalus acanthias*. *Marine Biology*, **6**: 248–255.

Reference: *Biol. Bull.*, **148**: 320–332. (April 1975)

INSECT HORMONES AND BIOANALOGUES: THEIR EFFECT ON RESPIRATORY METABOLISM IN *DERMESTES VULPINUS* L. (COLEOPTERA)

KAREL SLÁMA AND MAGDALENA HODKOVÁ

Institute of Entomology, Czechoslovak Academy of Sciences, Prague

Postembryonic development of insects proceeds as distinctive cycles characterized by specific changes in structure and function. From particular physiological changes and hormonal conditions we can distinguish several categories of developmental cycles: (i) larval-larval molt cycles, (ii) larval-pupal and larval-adult molt cycles, (iii) pupal-adult molt cycles in the Endopterygota, (iv) reproduction cycles in adult females and, (v) a combination of molt and reproduction cycles in the Apterygota. It has been recognized earlier (Sláma, 1968) that in addition to growth and developmental patterns, each of the above categories can also be characterized by certain specific type of total body metabolism. By altering hormonal conditions it has been possible to induce prematurely or to postpone the appearance of the given developmental cycles. And, without respect to age or absolute size of the body, the specific patterns in total body metabolism have also been correspondingly transposed (hand-in-hand with the transposition of the cycle in question). This has been documented in studies of juvenile hormone-induced transposition of the larval and adult molt cycles in Exopterygota (Sláma, 1964, 1965, 1968, 1971; Sláma, Romaňuk and Šorm, 1974) and by observations on the larval, pupal, and adult molt cycles in Endopterygota (Sehnal and Sláma, 1966).

In the present paper we have extended these studies on the relationships between hormones, growth, morphogenesis, and respiratory metabolism to larvae and pupae of a coleopteran of the family Dermestidae. The advantages of this material for metabolic investigations are shown by the fact that it was possible to delay the pupal-adult molt cycle by one or more extra pupal cycles simply by treatment with the juvenile hormone bioanalogues (juvenoids). Moreover, it was possible to induce extra larval and larval-pupal molt cycles by injections of bioanalogues of the prothoracic gland hormone (ecdysoids).

MATERIALS AND METHODS

The larvae and adults of *D. vulpinus* were reared in glass jars at 27° C and 18 hr photophase. They were fed dried calf viscera and supplied with water in cotton plugged glass vials. All the non-feeding stages were incubated at 27° C in Petri dishes which were provided with a source of moisture.

Oxygen consumption was measured with a Warburg apparatus using conventional techniques (Sláma, 1960). The respiratory vessels were cylindrical and without side arms. Their internal volume was approximately 10 ml. The values of O_2 consumption presented in the figures are averages of 3 to 5 successive read-

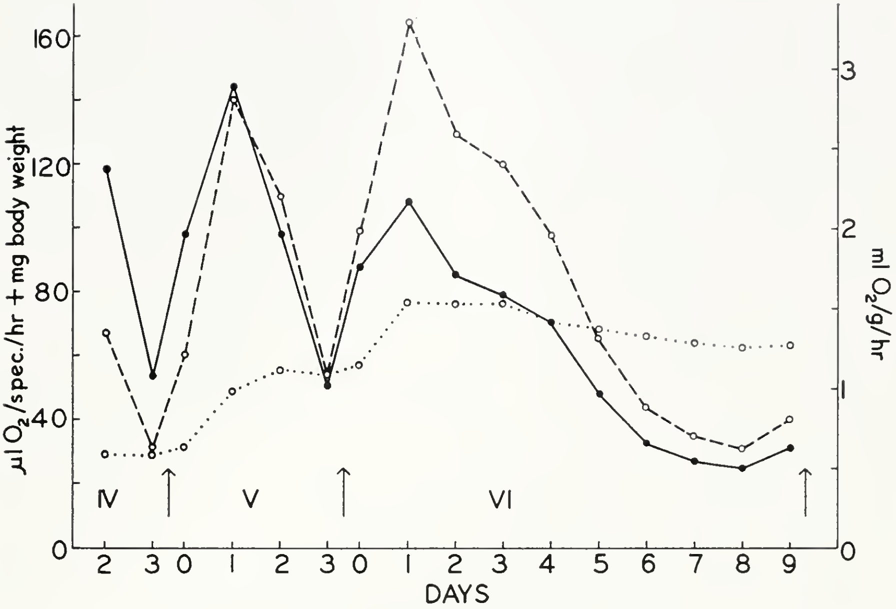


FIGURE 1. Changes in average body weight (dotted line), O_2 consumption in μl/hr/specimen (broken line), and O_2 consumption in ml/gram live weight/hr (full line) during the 4th, 5th, and last (6th) larval instars of *D. vulpinus.* The arrows indicate ecdyses ($n = 12$ specimens).

ings on each of the individually measured specimens, the number of which (n) is indicated in the legend. Due to considerable changes in body weight we have indicated O_2 consumption values both per specimen and per gram of fresh body weight in all the feeding stages. However, in the non-feeding stages where the body weight undergoes only slight and more or less constant diminution we have related the O_2 consumption values per gram of the initial weight only. The standard deviation from the mean values of Q_{O_2} in the feeding stages (Figs. 1 and 2) was around ± 30 per cent. In the non-feeding and immobile specimens (Figs. 3 to 7) the individual O_2 consumption curves of each specimen had parallel courses. There were extremely small deviations from the mean values (usually not exceeding ± 20 per cent). The deviations were more or less constant in each specimen suggesting that they were merely due to different size of the body and due to different content of reserve materials which take no active part in metabolism.

Out of a large number of juvenile hormone analogues we selected for the experiments with *Dermestes* an ethyl ester of 3, 7, 11-trimethyl, 11-chloro, 2-dodecenic acid. This juvenoid was highly active in topical applications and it appeared to be relatively stable in the body. The ID-50 Morph. unit of juvenile activity of this compound in the pupal assay on *D. vulpinus* is approximately 5/μg per specimen (Sláma *et al.*, 1974). In all cases topical application in 1/μl of acetone was used. Ecdysterone (Polypodine A isolated from *Polypodium* by Dr. J. Jizba of the Czechoslovak Academy of Sciences) was injected into the body cavity in 1/μl of 10 per cent ethanolic Ringer solution.

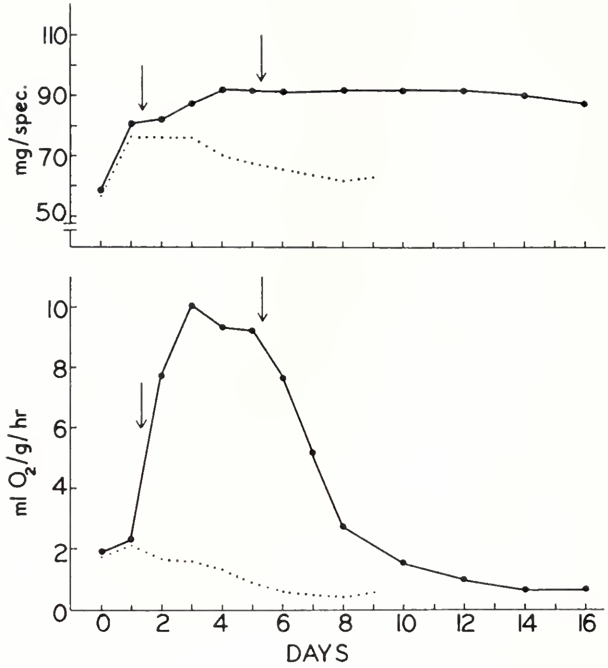


FIGURE 2. Changes in the body weight (above) and O_2 consumption (below) of last instar larvae treated with 50/μg/specimen of the juvenoid at the moments indicated by arrows (full line; n = 9 specimens). The dotted line shows comparison with untreated larvae.

RESULTS

Growth and respiratory metabolism in normal larvae

It is well known that changes associated with growth have a profound influence on larval metabolism. We have, therefore, recorded the changes in larval body weight which are more or less correlated with the rate of feeding and excretion. The results in Figure 1 show that the largest daily increments in body weight occur during the first day after each larval ecdysis. This is connected with intensive feeding activity at this time. A maximum body weight of the whole post-embryonic development is reached approximately at day 3 of the last (6th) larval instar. After that the weight successively diminishes throughout the rest of the last larval instar as well as during the whole metamorphosis period. Feeding is completely abolished from day 5 of the last larval instar.

In the penultimate (5th) larval instar the rate of O_2 consumption undergoes specific alterations which seem to be common for the larval-larval molt cycles. They have been found in the young larval instars of many unrelated insects by a number of authors (*cf.* Sláma, 1960; Sehnal and Sláma, 1966). It can be observed in Figure 1 that a common pattern of these metabolic changes is a relatively steep rise in O_2 consumption rate during the initial intensive feeding period, a peak in Q_{O_2} at about the middle of the inter-ecdysial period and, a subsequent fall of the metabolic activity towards the next ecdysis. This type of metabolic change can be briefly characterized as a reciprocal U-shaped course.

In the final, *i.e.* 6th larval instar, the course of O_2 consumption (Fig. 1) is somewhat different. Though the initial rise of O_2 consumption during the first day after ecdysis is present, the intensity of respiratory metabolism progressively diminishes, following a U-shaped pattern towards pupation. This type of metabolic cycle also seems to be widespread among last larval instars of many Endopterygote and Exopterygote insects, as discussed below.

Hypermetabolism induced by juvenoids in the last larval instar

In many Endopterygote insects it is extremely difficult or impossible to induce extra larval molts by application of juvenoids to the last instar larvae. This problem has been analyzed elsewhere (Sláma *et al.*, 1974). So also in *D. vulpinus* it has been so far impossible to cause the extra-larval instars or even larval-pupal intermediates by any available kind and amount of juvenoids. Out of all the morphological effects often connected with this treatment we observed only an occasional formation of pupae possessing larval cuticle on the distal ends of the legs, palpi, and antennae. The treated last instar larvae have always pupated after a considerable delay. The delay was proportional within certain limits to the dose of juvenoid and to number of applications, and it was well correlated with the specific juvenile hormone activity of various juvenoids as revealed by morphological assays on pupae of the same species (ID-50 Morph. units).

In addition, the above mentioned delay of pupation by juvenoids was always associated with enormously increased rate of feeding and excretion. The feeding period was prolonged from 5 to 10 or more days and the amount of excrement produced in daily intervals was drastically enhanced. Finally, when the affected larvae ultimately pupated, the pupae were significantly heavier than the normal ones (approximately 80 mg/specimen in contrast to about 65 mg of normal pupae). More importantly, they developed almost invariably into extra pupal instars or into adultoids.

One of the most spectacular physiological effects of juvenoids at the onset of the last larval instar of this species is an enormous increase in rate of O_2 consumption (see Fig. 2B). Indeed, treated larvae have consumed as much as 10 ml O_2 per gram live weight per hr and have maintained this extraordinarily elevated rate for several days. The degree of stimulation is unusual even among insects which often have high respiratory rates in comparison with other animals. Additional experiments, including tests of other vessel sizes and KOH concentrations, confirmed unequivocally that we were not dealing with any kind of artifact. We have thus discovered an unusual and specific effect of juvenoids for which we suggest the term "hypermetabolism".

Further studies revealed that the duration of hypermetabolism is limited. Even when juvenoids were continuously administered in excessive amounts, pupation was not delayed beyond certain time limits. The decline of O_2 consumption rate after several days of hypermetabolism could not be prevented by renewed juvenoid treatments, as is evident from Figure 2B. Moreover, hypermetabolism can be induced only when juvenoids are applied during the feeding period, *i.e.*, from ecdysis to the 5th or 6th day thereafter. Later treatments of the non-feeding last instar larvae had no effect on O_2 consumption of the prepupal stages.

The respiratory quotients determined by the indirect Warburg method revealed somewhat lower ratios in hypermetabolic larvae (0.719) in comparison with the equally old untreated larvae (0.755). This suggests that simultaneously with the almost 10-fold increase of O_2 consumption during hypermetabolism, the output of CO_2 was also enormously increased.

Dependence of hypermetabolism on nutrition

Preliminary experiments revealed that hypermetabolism was seriously limited or absent when the treated larvae were deprived of food. We have now performed another series of experiments using non-feeding and immobile larvae which had been ligated behind the head capsule. According to Sláma *et al.* (1974) the larvae of *D. vuplinus* can be ligated at any time in the 6th instar and still develop into headless pupae and adults. Moreover, when treated with juvenoids the ligated larvae were subjected to the same inhibition of metamorphosis as were normal feeding larvae. It was therefore advantageous to use ligated larvae for O_2 consumption measurements because variations due to differences in locomotory activity and food digestion were thereby avoided.

Figure 3 presents a comparison of O_2 consumption curves of normal last instar larvae and of individuals ligated shortly after ecdysis. In both instances the larvae

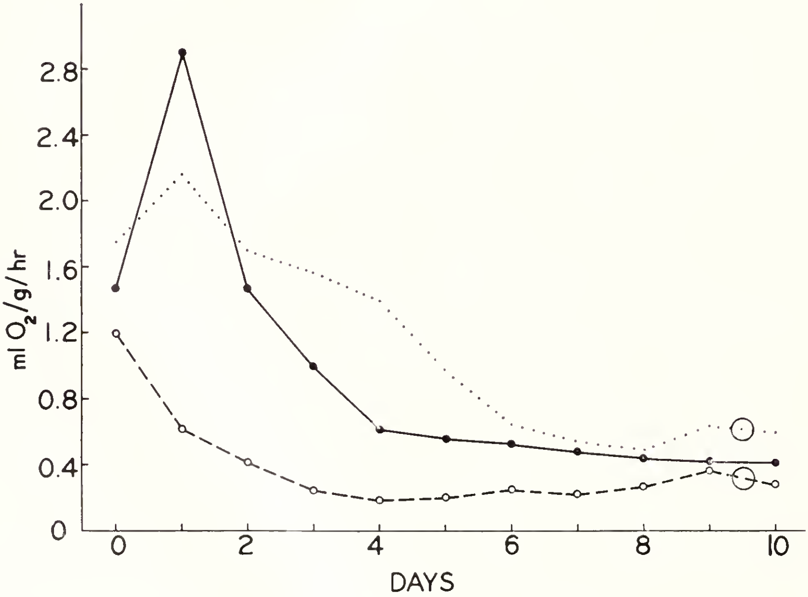


FIGURE 3. O_2 consumption of the last instar larvae which were ligated behind the head just after ecdysis (broken line; n = 5 specimens) or ligated and simultaneously treated with 50/μg/specimen of the juvenoid (full line; n = 6 specimens). The dotted line is taken from Fig. 1 and shows O_2 consumption of normal feeding larvae. Small rings indicate ecdysis or cryptoecdysis, respectively.

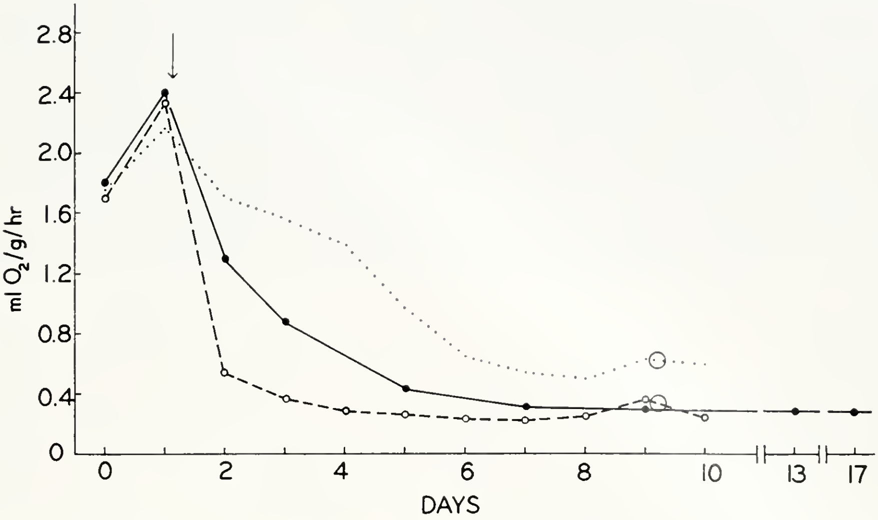


FIGURE 4. The same as in Fig. 3 with exception that the ligatures and juvenoid treatments were made after one day of feeding, as indicated by an arrow (full line; n = 10 specimens; broken line; n = 5 specimens).

developed into pupae after 9 to 10 days. The relatively low rates of O_2 consumption of the ligated larvae show that the processes of larval-pupal transformation alone have considerably smaller energetic requirements than do locomotion and food metabolism. The remaining curve in Figure 3 show the course of O_2 consumption in ligated larvae treated with juvenoid. Except for the first day after treatment their rates of O_2 consumption were less than that of normal feeding larvae. These ligated and treated larvae with completely inhibited metamorphosis maintain a higher metabolic rate than the untreated ligated controls. This suggests that juvenoids may induce certain metabolic changes even in the non-feeding larvae. However, the relative intensity of these metabolic changes is only a small portion of that encountered after hormonal treatment of feeding individuals.

Similar measurements on larvae that were ligated and treated with juvenoid after one day of feeding show basically the same relationships as have been described in the former experiment (*cf.* Figs. 3 and 4). Due to an increased content of reserve materials in the body the larvae which were ligated and treated with juvenoid after one day of feeding had a considerably improved rate of survival. They could be stored for several weeks without developmental changes. These dauerlarvae were used for most experiments with ecdysterone (see below). These results, and other evidence which will be published elsewhere, suggest that most but not all of the hypermetabolism is clearly dependent on the presence of metabolic substrates derived from the ingested food.

Juvenoid effects on metabolism during the pupal-adult transformation

As in most Endopterygote insects, the O_2 consumption rate of *D. vulpinus* followed a typical U-shaped metabolic curve which is characteristic for metamorphosis. At the time of pupal ecdysis (day −1 to 0 in Fig. 5) there occurs a temporary small peak in O_2 consumption which is associated with ecdysial functions. Approximately between day 1 and 2 there occurs adult apolysis in the untreated control pupae. This is followed by pharate adult development which culminates by adult ecdysis at day 7. More important for our study are newly found changes in O_2 consumption which reveal the course of metabolic processes during development of two extra pupal instars. This developmental feature was achieved by a single topical treatment of prepupae with large doses (100 μg per specimen) of the juvenoid. The results in Figure 5 show that the extra pupal molt cycles bring about specific modification of the normal U-shaped metabolic curve. This may be associated with a different type of growth among the tissues. While the untreated group of pupae underwent a pharate adult development from day 1 to day 7, the treated group of pupae underwent development simultaneously without morphogenesis resulting in the formation of first extra pupal instar at day 5 (this stage is usually referred to as second pupa or deuteropupa). Formation of the first extra-pupal instar is then followed by development of the second extra-pupal instar the cuticle of which is fully formed around the day 11.

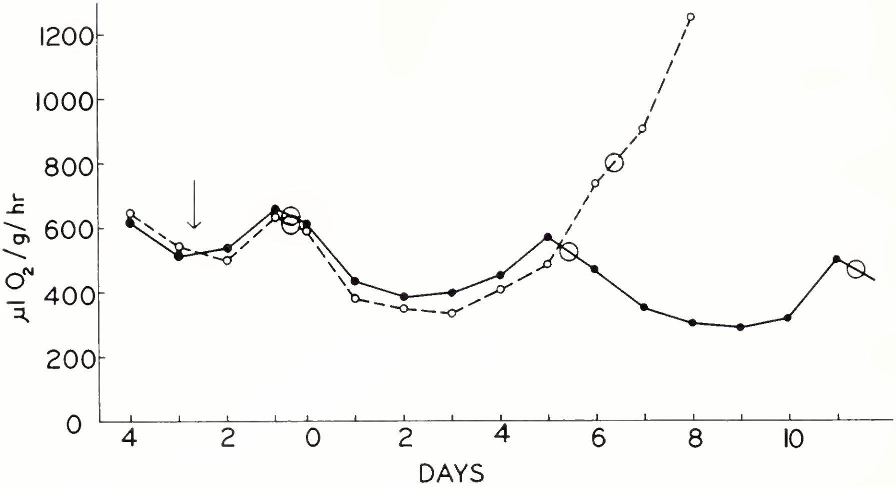


FIGURE 5. Changes in O_2 consumption rate during the prepupal period, in the pupal instar, and at the beginning of adult life in untreated controls (broken line; n = 10 specimens). Small rings on the curve indicate the moments of larval-pupal and pupal-adult ecdyses. Full line (n = 14 specimens) indicates changes in O_2 consumption rate during development of two successive extra-pupal instars induced by a single topical treatment with 100/μg of juvenoid per specimen at the moment indicated by arrow. Small rings on the curve indicate larval-pupal ecdysis, cryptoecdysis of the first extra-pupal instar and cryptoecdysis of the second extra-pupal instar.

The first and second supernumerary or extra-pupal instars had perfectly developed pupal epidermal patterns. The extra-pupal cuticles were fully formed one or two days earlier than the adult cuticle in untreated controls. They were well sclerotized and pigmented and the old endocuticle of the previous instars was regularly digested. However, in spite of the presence of these typical symptoms of ecdysis, the actual act of eclosion from the old exuvia never occurred in any of these stationary pupal-pupal molts.

In individual O_2 consumption curves for each specimen, we have observed that the mentioned modification of the U-shaped metabolic pattern appears also in a few instances where still a third extra-pupal development (not shown in Fig. 5) has taken place. We thus induced a series of several pupal-pupal molts with inhibited morphogenesis which were analogous to larval-larval molts as seen in normal development. However, in contrast to the larval molt cycles the induced repetitions of pupal molts were not connected with somatic growth.

Metabolism during the ecdysterone-induced molt cycles

In the feeding period of the last larval instar, ecdysterone (10 μg/specimen) caused more or less opposite effects to juvenoids. For instance, juvenoids increased the rate of feeding and stimulated hypermetabolism while ecdysterone suppressed or completely abolished feeding and reduced metabolic rate. It is possible that such an inhibitory effect of ecdysterone is partly related to hyperecdysonism (*cf.* Williams, 1968). In contrast to juvenoid applications, all larvae injected with these large doses of ecdysterone underwent precocious molt in 3 days.

The experiments with ecdysterone in the feeding or starved larvae did not allow us to identify an exact proportion of the metabolic intensity which would be attributed to the induced molt cycles. This was caused by relatively large variations in O_2 consumption due to differences in feeding and locomotory activity. Our attention was centered on immobile last instar larvae with inhibited metamorphosis. These were obtained from larvae that had been ligated and simultaneously treated with the juvenoid (50 μg/specimen) after one day of feeding. From the moment of juvenoid application the process of metamorphosis was completely inhibited and the larvae remained for weeks at the morphogenetic stage corresponding to the one-day-old last instar larvae. The initial course of O_2 consumption of these ligated dauerlarvae has been given in Figure 4. Approximately 15-day-old dauerlarvae maintained a very steady rate of O_2 consumption. This allowed one to distingush accurately even small changes in O_2 consumption.

Injections of small amounts of ecdysterone into 15-day-old dauerlarvae restored the larval-pupal molt cycles. Approximately 6 to 7 days after injection there appeared morphologically perfect headless pupae with fully pigmented pupal cuticle (Fig. 6A). After injections the rate of O_2 consumption was increased but this was later followed by a U-shaped curve of O_2 consumption prior to pupation. This metabolic pattern induced by small amounts of ecdysterone (0.5 μg/specimen) is similar to that found before pupation in the normal development (*cf.* Figs. 1 and 6A). The controls injected with solvent did not develop at all. They exhibited an injury effect on O_2 consumption which did not last for more than about 24 hr.

When injected with large doses of ecdysterone (10 μg/specimen), all the dauerlarvae underwent an extra-larval molt in 3 days. The extra larvae showed reduced bristles and incomplete dark larval pigmentation on some parts of the body. It is worthwhile to note that the same effect was also obtained when ecdysterone was directly injected into one-day-old normal last instar larvae. Some of the extra larvae continued to develop into morphologically perfect but headless

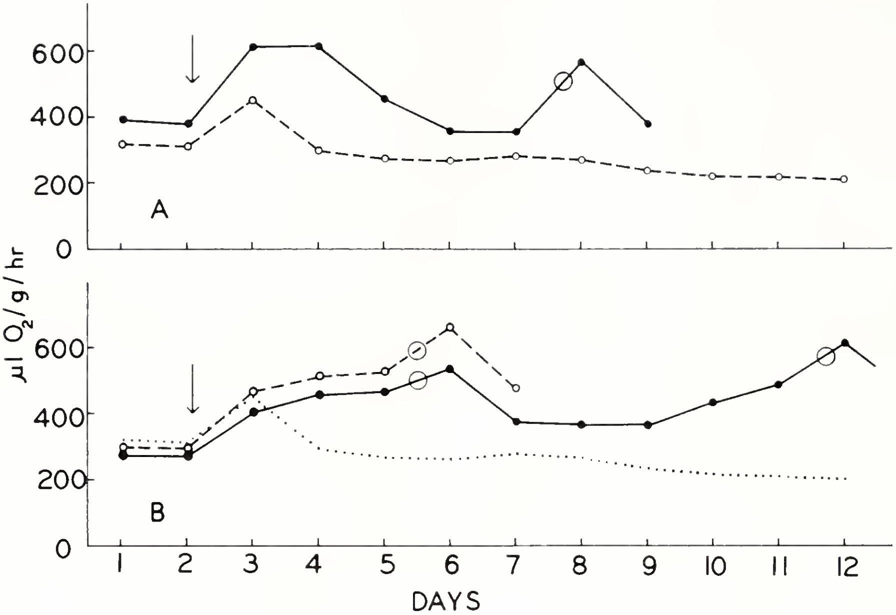


FIGURE 6. Effect of ecdysterone on O_2 consumption of the dauerlarvae. These were obtained by ligaturing behind the head and by topical treatment with 50/μg/specimen of the juvenoid in the one-day-old last instar larvae. The injections of ecdysterone, which are indicated by arrows, were made when the dauerlarvae were 15 to 20 days old. **A** shows the O_2 consumption of specimens injected with 0.5/μg of ecdysterone. These developed into headless pupae after 6–7 days (full line; n = 5 specimens out of 10 individual O_2 consumption curves). Small ring indicates larval-pupal cryptoecdysis. Broken line shows O_2 consumption of the controls injected with equal amounts of the solvent solution (n = specimens). **B** shows the O_2 consumption of specimens which received 10/μg of ecdysterone. Broken line (n = 15 specimens) represents larvae which exhibited extra-larval cryptoecdysis (indicated by small ring) and died afterwards. Full line (n = 5 specimens out of 10 individual curves) represents larvae which exhibited extra-larval cryptoecdysis and then pupal cryptoecdysis which is indicated by second ring. The dotted line is taken from Figure 6A and shows O_2 consumption of the controls.

pupae. As seen in Figure 6B, there was no remarkable difference between the injury response of controls and the ecdysterone injected larvae during the initial 24 hr period. Later, at the 2nd and 3rd day when the injury metabolism has faded out, the dauerlarvae injected with ecdysterone respired at about twice the rate of the controls. And there appeared also a small peak in O_2 consumption indicating unsuccessful ecdysis. Specimens which developed further into pupae after having experienced the extra-larval molt exhibited a similar type of the U-shaped metabolic course as in Figures 1, 3 and 6B.

It may be anticipated that differences in O_2 consumption between the controls with completely inhibited morphogenesis and the ecdysterone injected specimens undergoing extra-larval or larval-pupal molts would indicate approximately the amount of metabolic energy required for performance of the respective molt cycles. Our results have demonstrated that in relation to certain physiological functions such as are food digestion and intensive locomotion, the energetic requirements for the morphogenetic process alone are relatively minor. This conclusion is supported by the drastic changes in respiratory metabolism which take place during the 3-day larval-larval molt cycle in the growing 5th instar larvae (*cf.* Fig. 1) in contrast to a relatively small increase in metabolic intensity found in the ecdysterone induced 3-day larval-larval molt cycles which take place in the non-feeding ligated dauerlarvae (Fig. 6B).

Because our measurements were performed in daily intervals they would not record any possible short-term effects of ecdysterone on O_2 consumption. For this reason we performed an additional experiment with the injections of large doses of ecdysterone into the above described dauerlarvae. The O_2 consumption rate was then measured in successive one hr intervals during 24 hrs. The results are shown in Figure 7. It appears that the ecdysterone treated specimens have a slightly elevated respiratory rate during the initial 5 hr period after the injection. We are not certain, however, whether this is a specific response to ecdysterone or merely a result of variability in responses to epidermal injury. Somewhat later, from 7 hr after injections, the individual variations in O_2 consumption diminished. Subsequently, the ecdysterone treated specimens exhibited slow but continuous increase in O_2 consumption rate over the control level.

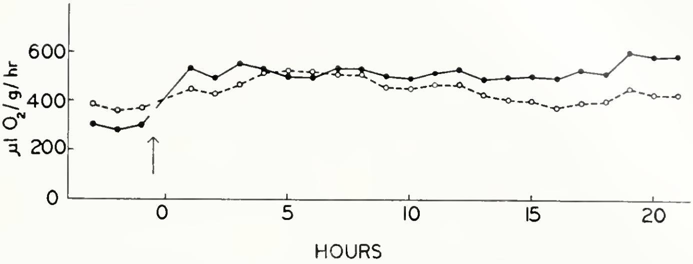


FIGURE 7. Same as in Figure 6B except that O_2 consumption readings were taken in 60 min intervals (full line; n = 8 specimens). Broken line (n = 5 specimens) shows O_2 consumption of the controls injected with the solvent alone. The arrow indicates the moment of injection.

DISCUSSION

Several authors studying the effect of hormones on respiratory metabolism in insects came to the conclusion that the effects were indirect depending on the degree of morphological and physiological changes induced in the reacting tissues (Pflugfelder, 1952; Neugebauer, 1961; Novák and Sláma, 1964, 1965, 1968, 1971; Lüscher and Leuthold, 1965; Wigglesworth, 1965b; Sehnal and Sláma, 1966). Our results with the bioanalogues of insect hormones are in full accord with this conclusion. We have demonstrated that juvenoids can cause enormous increases of respiratory metabolism when applied at certain periods in insect development whereas they are ineffective or cause completely different metabolic responses when applied at some other developmental period. Similarly, ecdysterone has a depressive metabolic influence when injected into feeding larvae although it stimulates metabolism in connection with induced molt cycles.

The results obtained in *D. vulpinus* also support earlier assumptions (Sláma, 1968, 1971; Sehnal and Sláma, 1966; Sláma *et al.*, 1974) that each of the hormonally conditioned developmental cycles in insects is characterized by certain specific patterns of total body metabolism. A retrospective look into literature on metabolism during insect postembryonic development (Kuznetzov, 1953; Wigglesworth, 1965a) reveals that the basic type of metabolic changes described here for the larval-larval, larval-pupal, and pupal-adult molt cycles may be a rather common phenomenon among insects belonging to quite different taxonomic groups. It is obvious from the results that the quality and timing of action of the hormones are essential for determination of the respective category of the developmental and metabolic cycles. However, the present findings on *Dermestes* also indicate that the metabolic responses of the tissues are not solely dependent on the hormonal milieu but are also subject to different feed-back reactions which may be mediated by nutritional conditions, availability of endogenous metabolic substrates, ontogenetic stage of the cells, *etc*.

The modified U-shaped metabolic pattern found in the experimentally induced extra-pupal instars of *Dermestes* appears to be very similar to that observed by Gilbert and Schneiderman (1961) and Steen (1961) in saturniid pupae treated with Cecropia extracts containing juvenile hormone. In addition, similar modification of the U-shaped metabolic curve in metamosphosis of *Tenebrio* has been found by Schmialek and Drews (1965) and by Geyer, Herda and Schmialek (1968) after treatments with farnesol derivatives. In all these instances the main difference between the juvenile hormone induced extra-pupal development and the normal adult development was the absence of elevated respiration which normally occurred prior to adult eclosion. This metabolic increase is associated among other factors with an extensive development of imaginal thoracic musculature. Because growth and differentiation of adult thoracic muscles are inhibited during the extra-pupal development, we assume that the absence of adult musculature may well account for the mentioned differences in metabolism.

The most spectacular effect of hormone analogues in *Dermestes* is undoubtedly the hypermetabolism in the final larval instar. In normal development, the young larval-larval instars which are commonly believed to develop in the presence of a high titre of juvenile hormone, have as a rule much higher respiratory metabolism (per unit of live weight) than the last instar larvae. In *Galleria* for example, the extra-larval molt cycles induced by implantations of *corpora allata* also show increased rate of respiratory metabolism. In addition, the extra-larval development has shown a type of metabolic course similar to that normally found in the penultimate and younger larval instars (Sehnal and Sláma, 1966). In *Galleria*, however, the metabolic increase associated with the extra-larval development was relatively small compared with hypermetabolism in *Dermestes*, where the tremendous metabolic stimulation was not a result of an extra-larval molt cycle.

The values of respiratory quotients, which are close to 0.7 during hypermetabolism, suggest that oxygen-poor substrates such as are lipids are being metabolized. Provided that this can be confirmed by a direct lipid analysis it would then indicate that hypermetabolism in *Dermestes* may represent a reciprocal effect to allatectomy which is commonly associated with decreased lipid metabolism and their accumulation in the body (Engelmann, 1970).

Since most of the compounds with juvenile hormone activity are oxygen-poor isoprenoids rich in methylene groupings, it is also possible that these compounds will be increasingly metabolized during hypermetabolism. This idea is supported by our observations that feeding larvae which show hypermetabolic responses require up to 100 times larger doses of juvenoid for the 5-day delay in pupation than do ligated larvae where hypermetabolism does not occur. It seems that the body reacts at this critical period at the onset of metamorphosis to the unwanted presence of juvenile activity by mobilizing certain metabolic systems for its deactivation or excretion. We are convinced that the hypermetabolism constitutes a part of the physiological "anti-juvenile" mechanisms which are known to operate in the last larval instar of this species (Sláma *et al.*, 1974).

It has been reported recently (Whitmore, Gilbert and Ittycheriah, 1974) that juvenile hormone applied to saturniid pupae induces formation of carboxyesterase enzymes which in turn inactivate the juvenile hormone esters. At present we have no evidence to show that an increased ester hydrolysis (which also involves splitting of glycerides) would be associated with hypermetabolic responses to juvenoids in *Dermestes*.

In contrast to the extensive amount of data on various biochemical effects of ecdysoids, our knowledge concerning their effects on total body metabolism is mainly based on indirect evidence derived from metabolic changes in metamorphosis and postdiapause development. The results obtained with ecdysterone in *Dermestes* suggest that the mode of action of this hormone does not depend on an immediate metabolism stimulating effect. Its depressive effects on metabolism of feeding larvae can be explained by precocious secretion of the new cuticle during which process the larvae usually stop feeding and reduce locomotion. On the other hand, in the non-developing and immobile stages, with low basal metabolism of maintenance, ecdysterone clearly stimulates more or less pronounced elevation of the metabolic rate. Changes in metabolism that follow later after ecdysterone injections are determined by the type and extent of the morphogenetic events, *i.e.*, by the respective category of the developmental cycle in question.

SUMMARY

1. Treatment of the early last instar larvae with juvenoids caused enormous increase in respiratory metabolism which is referred to as hypermetabolism. During this process the larvae consumed as much as 10 ml of oxygen per gram live weight per hour. It is anticipated that hypermetabolism constitutes part of a physiological "anti-juvenile" mechanism in *Dermestes*. The effect is associated with considerably enhanced food consumption and excretion. The phenomenon was virtually absent when juvenoids were applied to non-feeding larvae or pupae.

2. Single treatment of prepupal stages with large doses of juvenoids induced the formation of several extra-pupal instars. Each of them exhibited a slightly modified type of the U-shaped metabolic course.

3. Ecdysterone caused an indirect inhibition of the total body metabolism in the feeding larvae. In the non-feeding, immobile dauerlarvae it slowly increased the metabolic rate over the low maintenance level. In connection with stimulation of the molt cycles by ecdysterone there were specific patterns in respiratory metabolism which corresponded to the larval-larval or larval-pupal development.

4. Both the hormonal bioanalogues, *i.e.*, juvenoids and ecdysterone, are believed to have an indirect effect on the total body metabolism. The effect depends on the quality and degree of morphological and physiological changes conditioned by the hormonal milieu and on certain feed-back reactions. It has been confirmed that each of the developmental cycles in insects, such as are larval-larval, larval-pupal, and pupal-adult molt cycles, can be also characterized by a specific pattern in the course of respiratory metabolism.

LITERATURE CITED

Engelmann, F., 1970. *The Physiology of Insect Reproduction.* Pergamon Press Inc., New York, 307 pp.

Geyer, A., G. Herda and P. Schmialek, 1968. Beeinflussung des Stoffwechsels von *Tenebrio molitor* (Coleoptera) durch Farnesylmethyläther während des Puppenstadiums. *Acta Entomol. Bohemoslov.*, **65**: 253–262.

Gilbert, L. I., and H. A. Schneiderman, 1961. Some aspects of insect metamorphosis. *Amer. Zool.*, **1**: 11–51.

Kuznetzov, N. Y., 1953. *Osnovy fiziologii nasekomych. Izdat.* Acad. Nauk SSSR, Volume II, Moscow, Leningrad, 402 pp.

Lüscher, M., and R. Leuthold, 1965. Über die hormonale Beeinflussung des respiratorischen Stoffwechsel bei der Schabe *Leucophaea maderae* (F.). *Rev. Suisse Zool.*, **72**: 618–623.

Neugebauer, W., 1961. Wirkung der Extirpation und Transplantation der Corpora allata auf den Sauerstoffverbrauch, die Eibildung und den Fettkörper von *Caraussius* (*Dixippus*) *morosus. Arch. Entwicklungsmech. Organismen*, **153**: 314–352.

Novák, V. J. A., and K. Sláma, 1962. The influence of juvenile hormone on the oxygen consumption of the last larval instar of *Pyrrhocoris apterus* L. *J. Insect Physiol.*, **8**: 145–153.

Pflugfelder, O., 1952. *Entwicklungsphysiologie der Insekten.* Akad. Verlag Geest & Portig K.-G., Leipzig, 490 pp.

Schmialek, P., and G. Drews, 1965. Wirkung von Farnesylmethyläther auf die Atmung von *Tenebrio molitor* Puppen. *Z. Naturforsch.*, **20b**: 214–215.

Sehnal, F., and K. Sláma, 1966. The effect of corpus allatum hormone on respiratory metabolism during larval development and metamorphosis of *Galleria mellonella* L. *J. Insect Physiol.*, **12**: 1333–1342.

Sláma, K., 1960. Oxygen consumption during the postembryonic development of *Pyrrhocoris apterus* (Heterometabola: Heteroptera) and its comparison with that of Holometabola. *Ann. Entomol. Soc. Amer.*, **53**: 606–610.

Sláma, K., 1964. Hormonal control of respiratory metabolism during growth, reproduction, and diapause in female adults of *Pyrrhocoris apterus* L. (Hemiptera). *J. Insect Physiol.*, **10**: 283–303.

Sláma, K., 1965. Effect of hormones on growth and respiratory metabolism in the larvae of *Pyrrhocoris apterus* L. (Hemiptera). *J. Insect Physiol.*, **11**: 113–122.

Sláma, K., 1968. Hormonal control of developmental and metabolic cycles in insects. *Gen. Comp. Endocrinol.*, **9**: 492–493.

Sláma, K., 1971. Hormonal control of metabolism in *Pyrrhocoris. Endocrinol. Exp.*, **5**: 85–90.

Sláma, K., M. Romaňuk and F. Šorm, 1974. *Insect hormones and Bioanalogues.* Springer Verlag Wien, New York, 477 pp.

Steen, J. B., 1961. The effect of juvenile hormone on the respiratory metabolism of silkworm pupae, as recorded with a new semi-micro device. *Acta Physiol. Scand.*, **51**: 275–282.

Whitmore, D. Jr., L. I. Gilbert and P. I. Ittycheriah, 1974. The origin of haemolymph carboxylesterases induced by the insect juvenile hormone. *Mol. Cel. Endocrin.*, **1**: 37–54.

Wigglesworth, V. B., 1965a. *The Principles of Insect Physiology.* [6th Edition], Methuen, London, 741 pp.

Wigglesworth, V. B., 1965b. The juvenile hormone. *Nature*, **208**: 522–524.

Williams, C. M., 1968. Ecdysone and ecdysone-analogues: their assay and action on diapausing pupae of the *Cynthia* silkworm. *Biol. Bull.*, **134**: 344–355.

Reference: *Biol. Bull.*, **148**: 333–343. (April 1975)

THE INFLUENCE OF LIGHT IN THE DEPTH REGULATION OF CRAB LARVAE[1]

STEPHEN D. SULKIN[2]

Department of Zoology, Duke University, Durham, North Carolina

Light has been described as the principal environmental factor controlling the vertical distribution of planktonic animals (see *e.g.*, Bainbridge, 1961; Thorson, 1964). Previously, the author has demonstrated that responses of larvae of two Xanthid crabs, *Leptodius floridanus* (Gibbes) and *Panopeus herbstii* Milne-Edwards, to gravity and hydrostatic pressure provide a depth regulatory mechanism in the absence of light (Sulkin, 1973).

In this study, experiments were conducted to determine the photic responses of larvae of the same two species. The results of these experiments and an assessment of the influence of light on depth regulation are presented here.

Materials and Methods

Ovigerous specimens of *L. floridanus* were obtained commercially throughout the year from Florida; ovigerous specimens of *P. herbstii* were collected along rock jetties and oyster shoals near Beaufort, North Carolina, from May until October.

Both species have four zoea stages and one megalopa. Rearing conditions were chosen on the basis of work done on *P. herbstii* by Costlow, Bookout and Monroe (1962). A temperature of 25° C and a salinity of 25‰ were selected as the combination best suited for successful development in both species. Larvae from each female were kept in mass culture in several large finger bowls. Cultures were maintained in constant temperature cabinets with a photoperiod of 12 hours of light. Larvae were transferred daily to clean bowls of filtered seawater and fed freshly-hatched *Artemia salina* nauplii.

To study photic responses, a sample of larvae was pipetted into an observation tank, which was then sealed and inserted into one of the two observation compartments in the control cabinet illustrated in Figure 1. The interior of the compartment was lined with styrofoam, painted flat black. The light source, a 150 W incandescent bulb, could be positioned at either end of the tank and aimed along its axis. A sheet of ground glass was inserted between the bulb and the observation compartment to diffuse the light. To eliminate the possibility of a temperature gradient, the observation compartments were insulated from the light sources by two Plexiglas plates, separated by an air space. A dim red back-light behind the observation compartments silhouetted the larvae. The entire cabinet could be positioned vertically, as shown in Figure 1, or horizontally.

The observation tank was constructed of 1.2 cm thick transparent Plexiglas, measuring 61 cm long with an internal cross sectional area of 6.45 cm^2. Based

---

[1] Contribution No. 596 from the Chesapeake Biological Laboratory, Natural Resources Institute, University of Maryland.

[2] Present address: Chesapeake Biological Laboratory, Solomons, Maryland 20688.

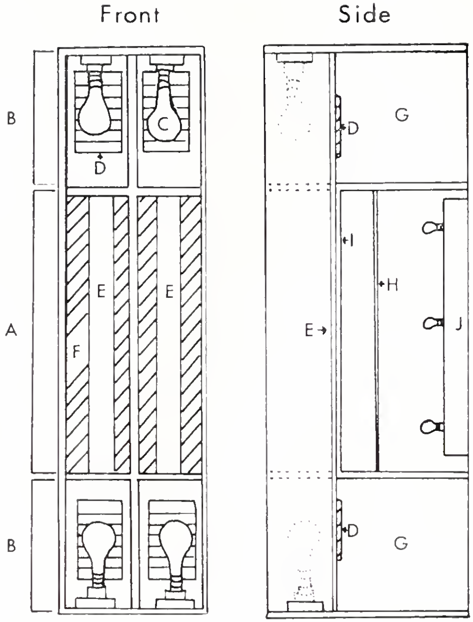


Figure 1. Light cabinet (figure not drawn to scale): A. chamber for observation tank; B. light chamber; C. light bulbs; D. ventilation outlets; E. red back-light window; F. styrofoam insulation; G. rear dead space ventilation area; H. frosted glass; I. red filter; J. back-light source.

upon published values for oxygen consumption of zoea and megalopa larvae (Vernberg and Costlow, 1966), the volume of the tank (500 ml) should have been sufficient to provide ample oxygen for the number of larvae used and the duration of the observations. Indeed, there was no observed change in behavior during the course of the experiments which could be attributed to oxygen deficit.

Specific details for each experiment are described with the results.

Results

Orientation to light

Light, at an intensity of 10 foot-candles (ft.-c), was aimed along the axis of the observation tank from one end. The tank was positioned horizontally so that gravity responses were isolated from those of light.

Figure 2 illustrates the primary orientations assumed by zoeae of all stages. Of the two primary orientations observed, that illustrated in Figure 2A was most common. When movement begins, the rostral spine and antennae are pointed down and away from the light. The dorsal spine is pointed toward the light with its main axis at an angle approximately 30° above the horizontal plane. The abdomen remains flexed. This orientation is accompanied by prolonged bursts of swimming.

In the other primary orientation (Figure 2B), movement proceeds backward, with the eyes facing away from the light and the rostral spine and antennae pointed

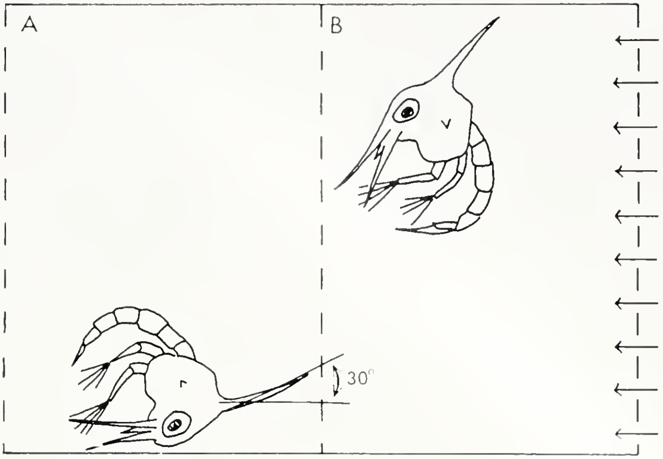


FIGURE 2. Orientation typical of zoea stages in horizontal light (light is coming from the right of the diagram as indicated by the arrows; the figure is not drawn to scale): A. position most frequently observed in horizontal light; B. position occasionally observed (see text for details).

down. The dorsal spine is held upright. The abdomen does not remain flexed, but flicks periodically. This behavior may be stimulated by a tendency to sink, since larvae assuming this orientation characteristically were located at the top of the horizontal tank.

The megalopa swims by the use of pleopods located on the extended abdomen. Periods of inactivity or slow crawling are interrupted by a "jump" up into the middle of the tank, followed by a series of looping movements which carry the megalopa toward the light.

Secondary orientation in response to light was tested in the following manner. Samples of each zoea stage and the megalopa of *L. floridanus* were placed in each of two horizontal observation tanks and allowed to disperse in darkness (dim red back-light). After 1 hour, light (10 ft.-c) was aimed along the axis of one of the tanks from one side. Larvae in the other tank remained in darkness. At intervals shown in Table I, the number of larvae in the half of the tank nearest the light source were counted, as were those in one half of the control tank (chosen at random). Results of repeated tests for each stage are shown in Table I as percent of total sample. In all four zoea stages and in the megalopa, net movement was toward the light (positive phototaxis).

A similar experiment was conducted on the first and fourth (last) zoea stages and the megalopa of *P. herbstii*. Based on the results with *L. floridanus*, a control was not deemed necessary. The results are shown in Table II. Again, net movement was toward the light (positive phototaxis).

Responses to changes in light intensity

The response of zoea larvae to various light intensities was measured in the following manner. A sample of larvae was pipetted into the observation tank which

TABLE I

Leptodius floridanus: *the per cent of total sample in half of observation tank nearest light source (experimental) or in randomly chosen half of tank in darkness (control) at specified time intervals.* (*N = total number of larvae tested in each case.*)

Stage of development	N		Time (min)										
			0	2	4	6	8	10	20	30	40	50	60
Zoea I	177	Control	51	50	50	50	53	53	50	51	50	47	50
	172	Experimental	49	78	84	88	90	90	92	94	94	93	93
Zoea II	57	Control	50	53	53	53	51	51	51	53	53	47	51
	59	Experimental	49	88	95	97	95	93	97	97	97	92	93
Zoea III	59	Control	51	54	51	53	51	47	51	56	49	49	49
	56	Experimental	48	86	91	91	93	95	96	93	96	95	95
Zoea IV	60	Control	50	45	45	43	43	47	47	45	47	47	48
	100	Experimental	49	86	93	95	96	95	96	96	95	94	94
Megalopa	45	Control	53	53	47	47	40	40	47	53	47	47	47
	45	Experimental	51	56	62	64	71	73	78	82	82	84	89

was then placed in the light-temperature cabinet in the horizontal position. The larvae were attracted to one end of the tank by a dim light. A light of known intensity was then aimed along the axis of the tank from the opposite end. As the larvae swam toward it, individuals were timed as they traversed a 10 cm long space marked off in the middle of the tank. Larvae pausing for more than 2 seconds were discounted. At each light intensity 50 individuals of *L. floridanus* (Stage I and Stage IV) and 30 of *P. herbstii* (Stage I) were timed. Swimming speed was calculated in cm/sec.

Four light intensities were tested. As measured by an irradiance meter at the end of the tank nearest the light source, they were 10 ft.-c, 30 ft.-c, 50 ft.-c, and 70 ft.-c. Higher intensities could not be tested in the apparatus because of the heat produced. Diminution of intensity along the 10 cm observation interval was negligible.

Data for each species or stage was subjected to the formal test of linearity (Li, 1964). Although the data from all three showed linearity, only in the first zoea stage of *P. herbstii* was the slope of the regression significantly different from

TABLE II

Panopeus herbstii: *the per cent of total sample in half of the observation tank nearest light source at specified time intervals.* (*N = total number of larvae tested in each case.*)

Stage of development	N	Time (min)										
		0	2	4	6	8	10	20	30	40	50	60
Zoea I	176	48	85	83	82	85	87	91	82	82	83	85
Zoea IV	96	49	77	81	85	83	87	90	92	89	91	93
Megalopa	52	50	52	58	62	64	70	74	74	74	81	81

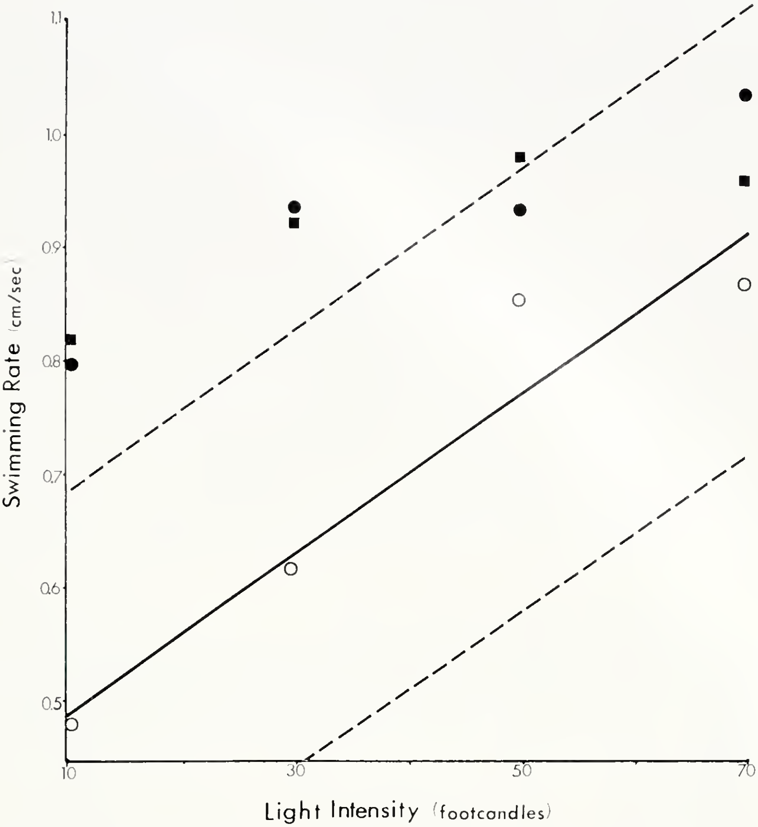


FIGURE 3. Regression of swimming rate as a function of light intensity in the first zoea stage of *P. herbstii*. The two parallel dashed lines represent one standard error to either side of the solid regression line. The points indicate mean swimming rates measured for each larval stage at each light intensity (*P. herbstii*, Stage I: open circles; *L. floridanus*, Stage I: solid circles; *L. floridanus*, Stage IV: solid squares.)

zero ($P < 0.025$). The line of regression and standard errors are shown in Figure 3. An increase in swimming rate with an increase in light intensity is clearly indicated in this stage. Although both the first and fourth zoea stages of *L. floridanus* showed slight increases in swimming rate with an increase in light intensity, the slopes were not statistically significant (Stage I = $0.05 < P < 0.10$; Stage IV = $P > 0.25$). The mean swimming rates for *L. floridanus* ranged from 0.80 to 1.04 cm/sec (Stage I) and from 0.82 to 0.97 cm/sec (Stage IV); mean swimming rates for *P. herbstii* (Stage I) ranged from 0.48 to 0.86 cm/sec (Fig. 3).

Vertical distribution in light from above

Samples of each zoea stage and the megalopa were placed in each of two horizontal observation tanks and allowed to disperse in darkness (dim red back-light). After 1 hour, the tanks were rotated slowly to the vertical position, and the number

TABLE III

Leptodius floridanus: *the per cent of total sample in the upper half of the observation tank at the specified time intervals during "lighted tank" experiments. Italicized values are those measured in the "dark tank" experiments. These values were taken from Sulkin, 1973, Table I, page 76. (N = total number of larvae tested in each case.)*

Stage of development	*N*	Time (min)										
		0	2	4	6	8	10	20	30	40	50	60
Zoea I	172	51	70	77	78	74	74	64	66	61	55	56
	181	*53*	*60*	*60*	*62*	*58*	*60*	*57*	*50*	*47*	*54*	*57*
Zoea II	52	50	58	60	63	69	71	65	63	63	71	69
	59	*49*	*29*	*24*	*27*	*27*	*29*	*34*	*37*	*41*	*41*	*42*
Zoea III	92	51	62	72	74	73	70	66	73	67	71	73
	99	*49*	*32*	*30*	*33*	*39*	*38*	*38*	*37*	*40*	*41*	*38*
Zoea IV	98	51	62	58	55	58	60	55	52	53	55	54
	98	*49*	*28*	*19*	*19*	*18*	*20*	*17*	*18*	*22*	*17*	*16*
Megalopa	45	49	29	33	33	31	33	31	31	31	31	31
	44	*50*	*9*	*7*	*7*	*7*	*5*	*0*	*0*	*0*	*0*	*0*

of larvae in the upper half of each tank was counted (time = 0). Larvae in one of the two tanks were then subjected to light (10 ft.-c) aimed down the axis of the tank from above; the other tank remained in darkness. Numbers of larvae in the upper half of each tank were counted at prescribed intervals. The results of the "dark tank" experiment have been reported in detail elsewhere (Sulkin, 1973; see discussion).

The "lighted tank" experiment was conducted only on larvae of *L. floridanus*. Results of repeated tests for each stage are shown in Table III as per cent of total sample. ("Dark tank" values are included for comparison. The values, in italics, were taken from Sulkin, 1973, Table I, page 76.) In the lighted tank, the first zoea stage showed a net upward movement, with values ranging from a high of 78% at the six minute interval to a low of 55% at 50 minutes. The second and third zoea stages also showed a net upward movement, with values ranging from 71% to 58% and from 74% to 62%, respectively. The fourth zoea stage showed little net movement, with values ranging from 62% to 52%. The megalopa reached an equilibrium at 31% after 20 minutes.

In order to assess the impact of light on vertical distribution, the values obtained in the "lighted tank" were compared with those in the "dark tank" at the time of termination of both experiments (60 min). The per cent increase of the former over the latter is shown in Table IV. Light exerts a negligible effect upon

TABLE IV

Leptodius floridanus: *the per cent increase in values obtained in the "lighted tank" experiment over those obtained in the "dark tank" experiment at the 60 min interval.*

Zoea I	Zoea II	Zoea III	Zoea IV	Megalopa
−1.8	64.2	92.1	237.5	∞

the net vertical distribution of the first zoea stage, but substantially increases the per cent of total sample in the upper half of the tank in each of the succeeding larval stages.

These vertical distribution values represent equilibria, with frequent exchange of larvae between the upper and lower halves of the tank.

Relative influence of light and gravity as orienting stimuli

The zoea stages of *L. floridanus* and *P. herbstii* have been shown here to be positively phototactic and have been reported previously to be negatively geotactic (Sulkin, 1973). The influence of light and gravity can be opposed experimentally if a sample of larvae in a vertically positioned tank is subjected to light aimed up from below ("experimental"). The relative strengths of the two stimuli can be assessed by comparing the vertical distribution under the experimental conditions just described with that obtained when gravity alone is the stimulus. The latter condition can be simulated in a vertically positioned tank in darkness ("control"). After a 1-hour exposure to each of the two conditions, the number of larvae in the bottom quarter of each tank was counted. The pooled results of five independent tests are shown in Table V and are expressed as per cent of the total sample.

TABLE V

Percentages of first stage zoeae found in the bottom quarter after the sample has been subjected to either the control or the experimental conditions for one hour. Total number of larvae subjected to each test are shown in parentheses. Five independent tests were conducted in each case.

Stimulus	*L. floridanus*	*P. herbstii*
Control*	43 (N = 179)	7 (N = 165)
Experimental**	94 (N = 152)	99 (N = 164)

* Tank vertical, no light; gravity only taxis clue.
** Tank vertical, light from below; gravity and light taxis clues present and opposed.

A greater per cent of larvae was present at the bottom when light was aimed up from below than when gravity alone was the orienting stimulus. It can be inferred from this data that light response dominates that of gravity in a majority of the first stage zoeae of *L. floridanus* and *P. herbstii*.

Since the megalopa of *L. floridanus* and *P. herbstii* are both positively phototactic (as reported here) and positively geotactic (Sulkin, 1973), the stimulating influences of light and gravity are opposed in nature. This situation can be simulated experimentally by shining light down from above ("experimental"). The impact of light upon the vertical migration of megalopa can be tested by comparing results obtained in the "experimental" condition with those obtained using a vertical tank in darkness ("control"). The per cent of megalopa present in the bottom quarter of control and experimental tank are shown in Table VI.

The application of light aimed down from above stimulated only 30% of *L. floridanus* and 10% of *P. herbstii* to swim up from the bottom. It is apparent that

TABLE VI

Percentages of megalopa found in the bottom quarter after the sample has been subjected to either the control or the experimental conditions for one hour. Total numbers of larvae subjected to each test are shown in parentheses. Three independent tests were conducted in each case.

Stimulus	*L. floridanus*	*P. herbstii*
Control*	100 (N = 44)	100 (N = 50)
Experimental**	70 (N = 45)	90 (N = 50)

* Tank vertical, no light; gravity only taxis clue.

** Tank vertical, light from above; gravity and light taxis clues present and opposed.

the positive gravity response is dominant in a majority of the megalopa of both species.

DISCUSSION

The use of light in experimental studies of behavior of aquatic animals is a subject of considerable controversy. In his review of light as a factor influencing the distribution of invertebrate larvae, Thorson (1964) examined the question. He concluded that no standard rules for determining experimental light conditions were possible because light penetration is so heavily dependent upon local hydrographic conditions. The choice of experimental intensities is governed ultimately by the hydrographic conditions associated with the species' habitat and by limitations imposed by the experimental design.

Larvae of both *L. floridanus* and *P. herbstii* are likely to be found in estuarine and nearshore coastal areas. In his study of crab larval distribution in the Newport River Estuary near Beaufort, North Carolina, Pinschmidt (1963) reported mean secchi disc readings of from 0.28 m to 1.56 m. Readings generally were lowest during the spring and summer, the time of greatest abundance of brachyuran larvae. A mean value of 0.46 m can be calculated for the period between May and October at Pinschmidt's station 3, located midway along the salinity gradient in the lower Newport River. To estimate the depth reached by 1% of ambient radiant energy, secchi disc depth can be multiplied by a factor of 3.5 (Holmes, 1970). Thus only 1% of ambient surface radiation is present at a depth of 1.6 m. Field studies have indicated that larvae of xanthid crabs are found in greatest abundance at depths which exceed 1.6 m by factors of from 2 to 9 (Bousfield, 1965; Dudley and Judy, 1971; Sandifer, 1973).

It thus appears that proper experimental procedure requires the use of very low intensities. A range of from 10 ft.-c to 70 ft.-c was chosen for this study to meet this criterion. By comparison, it is at the lower end of the range used by Bayne (1964) in his classic study of phototaxis in bivalve larvae.

The positive phototaxis described here for early zoeae of *L. floridanus* and *P. herbstii* confirms the observations reported by Spooner (1933), Foxon (1934), Christenson and McDermott (1958), and Herrnkind (1968) for other species. Specific reports on phototaxis in late zoeae and megalopa are rare. Welsh (1932) found that some larvae became photonegative as development proceeded in the mussel crab *Pinnotheres maculatas*. In contrast, the current study demonstrates that in the two species tested, late zoeae and the megalopa remain positively phototactic.

Both of the primary orientations reported here have been described by Welsh (1932) and Spooner (1933) for other zoea larvae. Contrary to these earlier reports, however, the present study indicates that the "forward" position illustrated in Figure 2A is most common. The location at the top of the horizontal tank characteristic of the "backward" primary orientation (Figure 2B) suggests that negative geotaxis and positive phototaxis combine in a small per cent of the larvae. When primary orientation is dominated by gravity, movement is upward. After the larvae have reached the top of the horizontal tank, they swim toward the light. The backward movement may be the only orientation possible which permits movement toward the light when the gravity stimulus is dominant.

Fraenkel and Gunn (1961) define "high kinesis" as an increase in activity which results from a high intensity of stimulus. By this definition, the response of first stage zoeae of *P. herbstii* to changes in light intensity can be termed high photokinesis. The comparative insensitivity of larvae of *L. floridanus* illustrates that there may be considerable variability in behavior among larvae of closely related species. The difference between these two species may be due to the apparent higher intrinsic rate of swimming in larvae of *L. floridanus* as compared to *P. herbstii*. *L. floridanus* larvae may be swimming at or near their physical capacity even at low light intensities.

The relative influence of light and gravity as taxis stimuli is of special significance in the megalopa. The dominant positive geotaxis shown by the megalopa is in conflict with the results reported by Rice (1966) for megalopa of *Macropipus sp.* and *Carcinus maenas,* both of which exhibited negative geotaxis. However, the apparent presence in this stage of conflicting orienting stimuli results in a dynamic situation which could cause considerable variation in response among different species. The specific responses of each species may be related to habits of the adult or to specific requirements for metamorphosis.

The author has suggested elsewhere that the responses of larvae of *L. floridanus* and *P. herbstii* to gravity and hydrostatic pressure, combined with characteristic passive sinking rates, provide a mechanism for depth regulation in the absence of light. Laboratory studies suggested that the four zoea stages and the megalopa of each species assume a differential vertical distribution, with succeeding stages showing a deeper net distribution (Sulkin, 1973).

The application of light at low intensities alters this basic pattern considerably. The response varies with each larval stage. While the net vertical distribution of the first zoea stage appears not to be influenced by light, the remaining stages show a much higher per cent of the sample in the upper half of the tank (Table IV).

Although a pattern of differential vertical distribution is retained, the relationships among the larval stages are different. The second and third stages may show a more shallow net distribution than does the first stage, although the fourth zoea stage and the megalopa are deeper. It is apparent, however, that the deeper position of late developmental stages is not due to a change in phototaxis as has been theorized for some other groups of larvae (Thorson, 1964).

Because light is such a variable parameter in the marine environment, its impact upon vertical distribution should be considered supplementary to the pattern shown by crab larvae in the absence of light. The more shallow net distribution of most stages when light is present indicates that an intrinsic mechanism for diurnal vertical migration is present. Experimental evidence thus indicates that larvae should move up toward the surface during the day and gradually disperse downward at night. Such a pattern has been reported in field studies of larvae of the lobster *Homarus americanus* (Templeman and Tibbo, 1945).

By scattering vertically, meropelagic larvae may encounter horizontal currents moving in different directions at different depths, thus increasing the potential for dispersal. Differential vertical distribution through ontogeny provides an intrinsic mechanism for enhancing vertical scattering during larval development. This study has demonstrated that the response of larvae to light superimposes upon the basic pattern an additional mechanism for vertical scattering; namely, diurnal vertical migration.

This work is part of a dissertation in partial fulfillment of the requirements for the Ph.D. degree at Duke University. The work was supported by Graduate Teaching Assistantships in the Department of Zoology at Duke University; by National Science Foundation Summer Traineeships at Duke University Marine Laboratory; and by a Summer Graduate School Award offered by Duke University. I am indebted to a number of members of the Departments of Zoology and Botany at Duke University for their assistance, especially my research advisor, Dr. C. G. Bookhout, for his direction and helpful criticism. I also wish to thank Dr. R. Scheltema for his editorial suggestions concerning this manuscript.

SUMMARY

1. Experiments were conducted to determine the phototaxis responses of each larval stage of two species of Xanthid crabs and to assess the influence of light on depth regulation.

2. Two primary orientations in response to light are described.

3. Secondary orientation in response to light results in positive phototaxis in all four zoea stages and the megalopa of each species.

4. In response to changes in light intensity, the first zoea stage of *P. herbstii* demonstrated high photokinesis. Although some increase in swimming rate in response to increased light intensity was noted in larvae of *L. floridanus,* particularly in the first zoea stage, the differences were not statistically significant. It is suggested that larvae of *L. floridanus* may swim at their capacity at low light intensities, in contrast to larvae of *P. herbstii.*

5. Light response dominates that of gravity in the first zoea stage; in the megalopa, gravity response dominates that of light in a majority of individuals.

6. Light aimed down the axis of the observation tank exerts a negligible effect upon the net vertical distribution of the first zoea stage. However, the net distribution of later larval stages is shallower in light than is the case in darkness.

7. The response of the larvae to light thus superimposes diurnal vertical migration upon the basic pattern of differential vertical distribution through ontogeny. These two characteristics of larval development will result in considerable vertical scattering during the dispersal phase.

LITERATURE CITED

Bainbridge, R., 1961. Migrations. Pages 431–463 in T. H. Waterman, Ed. *The physiology of Crustacea,* Vol. 2. Academic Press, New York.

Bayne, B. L., 1964. The responses of the larvae of *Mytilus edulis* L. to light and to gravity. *Oikos,* **15**: 162–174.

Bousfield, E. L., 1955. Ecological control of the occurrence of barnacles in the Miramichi Estuary. *Nat. Mus. Canada, Bulletin,* No. **137**: 69 pp.

Christenson, A. M., and J. J. McDermott, 1958. Life history and biology of the oyster crab *Pinnotheres ostreum* Say. *Biol. Bull.,* 114: 146–179.

Costlow, J. D., Jr., C. G. Bookhout and R. Monroe, 1962. Salinity-temperature effects on the larval development of the crab, *Panopeus herbstii* Milne-Edwards, reared in the laboratory. *Physiol. Zool.,* **35**: 79–93.

Dudley, D. L., and M. H. Judy, 1971. Occurrence of larval, juvenile, and mature crabs in the vicinity of Beaufort Inlet, North Carolina. *U. S. Dept. of Commerce, NOAA Technical Report NMFS* SSRF-637, 10 pp.

Foxon, G. E. H., 1934. Notes on the swimming methods and habits of certain crustacean larvae. *J. Mar. Biol. Ass. U.K.,* **19**: 829–849.

Fraenkel, G. S., and D. L. Gunn, 1961. *The Orientation of Animals.* Dover Publications, New York, N. Y., 376 pp.

Herrnkind, W. F., 1968. The breeding of *Uca pugilator* and mass rearing of the larvae with comments on the behavior of the larval and early crab stages. *Crustaceana,* Supplement No. **2**: 214–224.

Holmes, R. W., 1970. The Secchi disk in turbid coastal waters. *Limnol. Oceanogr.,* **15**: 688–694.

Li, J. C. R., 1964. *Statistical Inference I.* Edward Brothers, Inc., Ann Arbor, Michigan, 658 pp.

Pinschmidt, W., Jr., 1963. Distribution of crab larvae in relation to some environmental conditions in the Newport River Estuary, North Carolina. *Doctoral dissertation, Duke University,* 112 pp.

Rice, A. L., 1966. The orientation of pressure responses of some marine crustacea. *Proceedings of Symposium on Crustacea, Part III., Mar. Biol. Ass. India,* **1966**: 1124–1131.

Sandifer, P. A., 1973. Distribution and abundance of decapod crustacean larvae in the York River Estuary and adjacent lower Chesapeake Bay, Virginia, 1968–1969. *Ches. Sci.,* **14**: 235–257.

Spooner, G. M., 1933. Observations on the reactions of marine plankton to light. *J. Mar. Biol. Ass. U.K.,* **19**: 385–438.

Sulkin, S. D., 1973. Depth regulation of crab larvae in the absence of light. *J. Exp. Mar. Biol. Ecol.,* **13**: 73–82.

Templeman, W., and S. N. Tibbo, 1954. Lobster investigations in Newfoundland 1938 to 1941. *Newfoundland Dep. Nat. Resour. Res. Bull. (Fish),* No **16**: 1–98.

Thorson, G., 1964. Light as an ecological factor in the dispersal and settlement of marine bottom invertebrates. *Ophelia,* **1**: 167–208.

Vernberg, F. J., and J. D. Costlow, Jr., 1966. Studies on the physiological variation between tropical and temperature-zone fiddler crabs of the Genus *Uca.* IV. Oxygen consumption of larvae and young crabs reared in the laboratory. *Physiol. Zool.,* **39**: 36–52.

Welsh, J. H., 1932. Temperature and light as factors influencing the rate of swimming of the mussel crab, *Pinnotheres maculatas* Say. *Biol. Bull.,* **63**: 310–326.

Reference: *Biol. Bull.*, **148**: 344–356, (April 1975)

DOSE-RESPONSE EFFECTS OF GAMMA-RADIATION ON SEVERAL GROWTH FUNCTIONS OF *CAMPANULARIA FLEXUOSA*

JEROME F. WERMUTH[1] AND CHARLES D. BARNES

Department of Life Sciences, Indiana State University, Terre Haute, Indiana 47809

Many studies have been done on the effects of ionizing radiation on growth functions of both fresh-water and marine cnidaria. Puckett (1935) suppressed regeneration of hydranth positions in colonies of *Pennaria tiarella* with an x-ray dose of 10,000 roentgens. Daniel and Park (1951, 1953) produced considerable damage to the tentacles of the common brown hydra by x-irradiating the animals at a dose level of 9,600 roentgens. Wermuth and Barnes (1967, 1968) irradiated various portions of five-day-old colonies of *Campanularia flexuosa*. When starter material was irradiated at 81,000 roentgens, lead-shielded new stolons of the same colonies showed an increase in new growth over non-irradiated control colonies.

Brock and Strehler (1963) and Strehler (1964), employing x-ray doses of from 500 to 210,000 roentgens on ten-day-old colonies of *Campanularia flexuosa,* reported an increase in the life-span of the normally cyclically-regressing hydranths of this species with increasing doses of radiation. While these two papers do represent dose-response studies on a single growth function in this species, the data were not presented as dose-response curves. The other papers presented above involve only single-dose studies on the growth functions considered, and therefore cannot be used to produce baseline values for dose effects of ionizing radiation on the growth functions under study.

The present study is concerned with the dose-response effects of ionizing radiation, specifically gamma radiation, on several growth functions in the colonial hydroid *Campanularia flexuosa.* The growth functions monitored post-irradiation for this study include the following: (1) addition of new stolon material; (2) addition of hydranth positions to starters; (3) addition of uprights to stolons; (4) addition of hydranths to stolons post-irradiation; and (5) life-span of hydranths. Observations of certain qualitative phenomena associated with the gamma radiation are also presented. Finally, comparisons of the dose-response effects are noted, leading to some tentative conclusions concerning the nature of determination and differentiation underlying the morphogenetic events associated with these growth functions.

METHODS

Stock colonies of the E strain of *Campanularia flexuosa* were used in these experiments. These organisms were provided by Dr. Sears Crowell, Department of Zoology, Indiana University, Bloomington. Stock colonies, from which starter material for new colonies was obtained, were maintained in a 25-gallon Instant Ocean aquarium. The medium employed was Instant Ocean Synthetic Sea Salts. Both items were supplied by Aquarium Systems, Inc., 33208

---

[1] Permanent address: Purdue University, Calumet Campus, Hammond, Indiana 46323.

Lakeland Boulevard, East Lake, Ohio, 44094. Approximately 9 liters of Instant Ocean were removed once each week from the aquarium, and replaced with an equal amount of freshly prepared Instant Ocean.

Stock colonies were fed twice daily to build up sufficient biomass for providing starters for new colonies, and irregularly after that, but always several times each week. Experimental colonies were always fed twice daily, morning and evening, during the course of an experiment. In both cases, the food consisted of freshly hatched nauplii of *Artemia salina* (Metaframe San Francisco Bay Brand, obtainable from Wards Scientific Supply, Rochester, New York) filtered, washed once with fresh Instant Ocean, and resuspended after a second filtering in fresh Instant Ocean. New colonies were propagated according to the method of Crowell (1953); *i.e.,* new colonies were initiated by removing an upright, consisting of a stem and associated hydranths, from a well-established stock colony. The upright was then placed under a cotton thread previously tied the short way around an ordinary microscope slide. [See also Wermuth and Barnes (1969) for a diagram of this technique.] In practice, a stock colony is kept in one petri dish, and two already-marked and -tied slides are kept in another petri dish. All manipulations are done under a dissecting microscope. Upright removal is easily accomplished by using a small, chromed, curved surgical scissors. The curvature allows the investigator to see the upright being cut, without at the same time having an uncomfortable or awkward hold on the scissors. Further, the slightly opened legs of the curved scissors can form a small basket for catching the detached upright and moving it through the air-water interface to the second petri dish.

New colonies were started three to a slide. Five slides were placed in one 10-space standard staining rack. Each group of 15 new colonies were referred to as a series for record-keeping considerations. The staining racks were kept either in the Instant Ocean aquarium at a constant temperature (18° C) or in a walk-in cold room at 9° C. Temperatures were kept at a constant value throughout any particular experiment. While in the cold room, the staining racks containing the slides with growing colonies were immersed in staining dishes containing approximately 200 ml of Instant Ocean. The covered staining dishes were kept on a bacteriological shaker table modified to hold them; consequently, the colonies were subject to a gentle oscillation throughout the course of an experiment. For feeding purposes the staining racks were transferred one-by-one to a staining dish on the shaker table containing a thick suspension of brine shrimp nauplii for timed intervals of 3 minutes after which the staining rack was transferred back to its original staining dish. To minimize the effects of decaying egestate and metabolic end-products, the Instant Ocean medium in each staining dish was changed throughout the course of any experiment every two days; that is, at approximately 48-hour intervals.

Gamma radiation was delivered from a source consisting of one dozen rods of Co^{60} which could be raised or lowered from a well located in the center of a room approximately 2m by 2m. For purposes of irradiation, staining dishes containing colonies were placed at set distances from the edge of the well. The five slides were moved to the five staining rack slots closer to the radiation source, so that colonies were oriented with their long axes at right angles to the radiation. In other words, if an imaginary line were drawn from each end of a colony-containing slide to the source, a triangle rather than a straight line would result. Bunching the five colony-bearing slides at one end of a staining dish accomplished the following: (1) maintained the dose level of the slide furthest from the source as close as possible to that of the slide closest to the source, and (2) approximated the diameter of the vial containing the dosimetry reagent. Dosimetry was accomplished by placing a vial of HE-6 reagent along side of or on top of the staining dish at the level of the slides. The HE-6 dosimetry system is described by McLaughlin, Hussmann, Eisenlohr, and Chalkley (1971).

During the course of the irradiation, which in all cases was 80 minutes, control colonies were kept in a control room adjoining the source room. The temperature of the Instant Ocean medium in the staining dishes containing the colonies being irradiated rose no more than 3° C during the irradiation period, control colony Instant Ocean rose no more than 2° C during the same period. Immediately upon cessation of irradiation, the irradiated medium was discarded and fresh Instant Ocean was added to each staining dish.

Daily records of the growth functions mentioned above were kept for all experimental colonies. Daily records of the developmental condition of individual hydranths were also maintained.

RESULTS

Qualitatively, no immediate difference in the behavior or the appearance on irradiated and non-irradiated colonies of *Campanularia flexuosa* was observed. Hydranths continued to feed; stolons continued their outward growth. At 24 hours post-irradiation, changes in stolon growth and hydranth production by those colonies which had received 80 Krad were observed. Rarely did the starters of these colonies add more than one new hydranth position; in rare cases only did this hydranth position proceed beyond an obviously damaged pedicel stage. In those cases where a hydranth did proceed beyond this point, a small, skinny-looking hydranth with reduced numbers and sizes of tentacles was produced. These hydranths did possess the ability to capture brine shrimp nauplii, indicating the presence of nematocytes in their truncated tentacles. Hydranths which are in developmental stages beyond the pedicel will continue to differentiate into properly formed and functional hydranths which evenutally regress, but new hydranths are not regenerated from these positions.

Several cases of disruption of hydranth polarity by gamma radiation were observed. Normally, the long axis of the first hydranth of an upright develops at an obtuse angle relative to the long axis of the distal stolon; the long axis of the second hydranth emerges at a 90° angle to the long axis of the first, with subsequent hydranths emerging in alternate fashion, each one slightly farther away from the stolon; *i.e.,* higher up the upright. In a few cases it was observed that in new uprights which appeared post-irradiation, the first hydranth position emerged with its long axis directed away from the starter. In one case, two hydranth positions emerged at the same distance from the stolon, but 180° from each other on the upright.

Very high radiation levels (80 Krad) almost completely suppressed upright formation on stolons, but did not completely suppress stolon growth or extension itself. This combination of phenomena produced abnormally long stretches of distal stolon, *i.e.*, stolon beyond the last upright. Gamma-irradiated stolons never showed the "denting" of the coenosarc described by Wermuth and Barnes (1969) when colonies of *Campanularia flexuosa* were x-irradiated. On the other hand, the squaring or blunting of the normally-rounded stolon tip was observed in *Campanularia flexuosa* after exposure to both gamma radiation and x-irradiation.

Quantitative results of the dose-response studies are presented in the following tables and figures. These data are derived from two separate experiments conducted at two temperatures, 9° C and 18° C. The 9° C-series was maintained in individual staining dishes in a cold room; the 18° C-series was maintained in an Instant Ocean aquarium. The 9° C-series received gamma-radiation doses of 0, 16, 24, 40, and 80 Krad. Those of the 18° C series received gamma-radiation doses of 0, 9, 18, 30 and 80 Krad. The difference in dosage between the two experiments was the result of an unannounced change in the configuration of the source. The distance from the source, as measured from a set point, ranged from 8 through 38 cm in intervals of ten cm measured from the set point to the middle of the 5 slides in any one staining dish. Tables I and II are summaries

TABLE I

Summary of dose-response data for several growth function of gamma-irradiated colonies of Campanularia flexuosa. *Colonies were maintained at 9° C; irradiation, day 11 after starting colonies; experiment terminated on day 19.*

Growth Function	Dose Krad	Response ± S.D.	Number
		Avge. mm of stolon	of colonies
New stolon material added days 1–8 after irradiation	0	34.5 ± 6.2	12
	16	25.5 ± 7.9	13
	24	20.4 ± 5.1	11
	40	13.4 ± 3.6	12
	80	10.8 ± 4.1	13
		Avge. no. added	of colonies
Hydranth positions added to starters days 1–8 after irradiation	0	14.7 ± 4.9	15
	16	8.6 ± 3.6	14
	24	5.5 ± 2.4	13
	40	3.3 ± 1.4	15
	80	0.9 ± 0.6	15
		Avge. no. added	of colonies
Uprights added to stolons days 1–8 after irradiation	0	4.0 ± 0.6	12
	16	3.2 ± 0.7	13
	24	2.0 ± 0.7	11
	40	1.0 ± 0.5	13
	80	0.1 ± 0.2	14
		Avge. no. added	of colonies
Hydranth positions added to uprights on stolons, days 1–8 after irradiation	0	13.2 ± 2.2	12
	16	9.8 ± 3.4	13
	24	3.8 ± 1.6	11
	40	2.0 ± 1.0	13
	80	0.4 ± 0.5	14
		Days	of hydranths
Average life span, hydranths on new stolons	0	17.7 ± 1.3	32
	16	17.4 ± 0.9	52
	24	19.7 ± 2.3	46
	40	21.5 ± 2.1	45
	80	25.0 ± 2.9	29

TABLE II

Summary of dose-response data for several growth functions of gamma-irradiated colonies of Campanularia flexuosa. *Colonies were maintained at 18° C; irradiation, day 9 after starting colonies; experiment terminated on day 14.*

Growth function	Dose Krad	Response ± S.D.	Number
		Avge. mm of stolon	of colonies
New stolon material added days 1–5 after irradiation	0	14.0 ± 2.8	8
	9	12.1 ± 1.4	11
	18	5.8 ± 1.4	10
	30	4.1 ± 1.4	7
	80	4.5 ± 1.3	8
		Avge. no. added	of colonies
Hydranth positions added to starters days 1–5 after irradiation	0	8.1 ± 4.4	8
	9	4.9 ± 2.3	11
	18	1.4 ± 1.1	10
	30	1.7 ± 1.7	7
	80	0.1 ± 0.3	8
		Avge. no. added	of colonies
Uprights added to stolons days 1–5 after irradiation	0	4.9 ± 0.9	8
	9	4.1 ± 0.8	11
	18	1.6 ± 0.5	10
	30	1.0 ± 0.8	7
	80	1.1 ± 0.3	8
		Avge. no. added	of colonies
Hydranth positions added to uprights on stolons, days 1–5 after irradiation	0	21.8 ± 6.4	8
	9	11.6 ± 3.5	11
	18	3.8 ± 1.9	10
	30	1.3 ± 1.3	7
	80	1.4 ± 0.7	8
		Days	of hydranths
Average life span of hydranths on new stolons	0	7.2 ± 0.8	60
	9	7.4 ± 1.1	87
	18	8.1 ± 1.3	74
	30	8.4 ± 1.2	33
	80	8.9 ± 2.0	42
		Days	of hydranths
Average life span of hydranths on starters	0	7.1 ± 1.0	191
	9	7.5 ± 1.0	210
	18	8.0 ± 1.2	228
	30	8.3 ± 1.0	190
	80	8.4 ± 1.5	180

of the dose-response data for several growth functions of gamma-radiated colonies of *Campanularia flexuosa* from the two separate experiments described above. They contain information concerning the number of colonies involved in each study, and the number of hydranths monitored for their longevity. Only hydranths which had already appeared by the day of irradition were included in these studies. Data for the life span of hydranths on starters of the 9° C are not available. Note that the experiment for the 9° C colonies terminated 8 days after irradiation; for the 18° C colonies, 5 days after irradiation. Thus, 9° C colonies were irradiated on day 11 after starting of colonies; 18° C colonies were irradiated on day 9 after starting of colonies. Starting day was day 0.

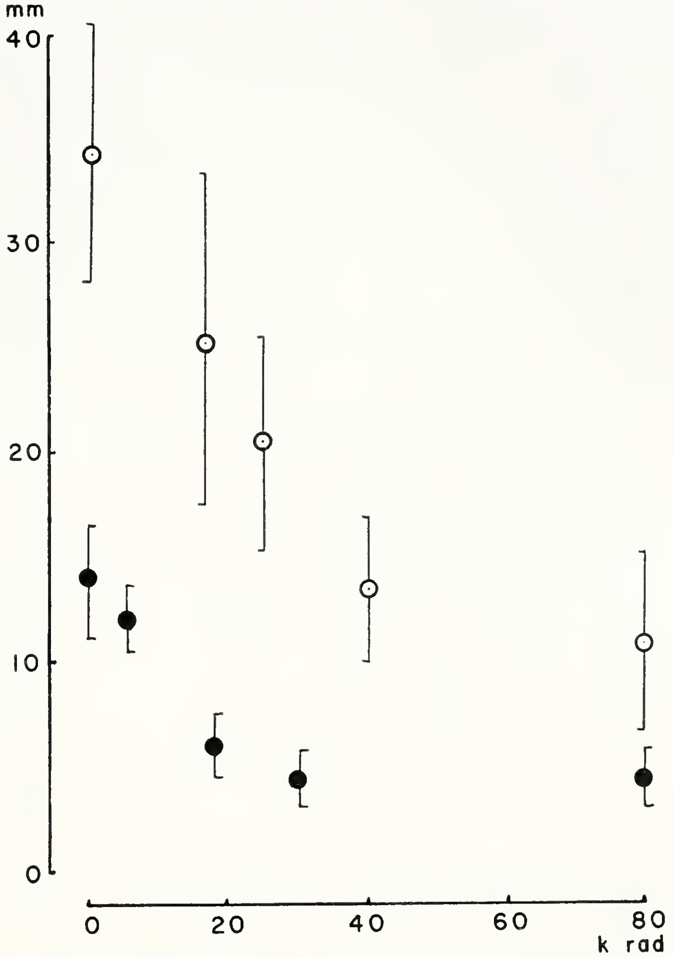


FIGURE 1. Average amount of new stolon material in mm added post-irradiation as a function of gamma-radiation dose in Krad. Open circles are 9° C colonies; closed circles are 18° C colonies. Vertical bars are one standard deviation above and below means.

Figures 1 through 6 present graphically the data presented in the summary tables. Figure 1 presents data on the average amount of new stolon material, in mm of linear growth as measured with the aid of an eyepiece micrometer. With increasing radiation dose, there is a linear decrease in the average amount of linear stolon growth, at least over the dose range from 0 to 30 Krad. An inflection in the curve is noted at that point, especially in the 18° C colonies, so that there is little or no difference in the response of those colonies which received 30 Krad and those that received 80 Krad. The inflection in the curve for the 9° C colonies is less evident but still obvious. A possible explanation of this difference will be presented later.

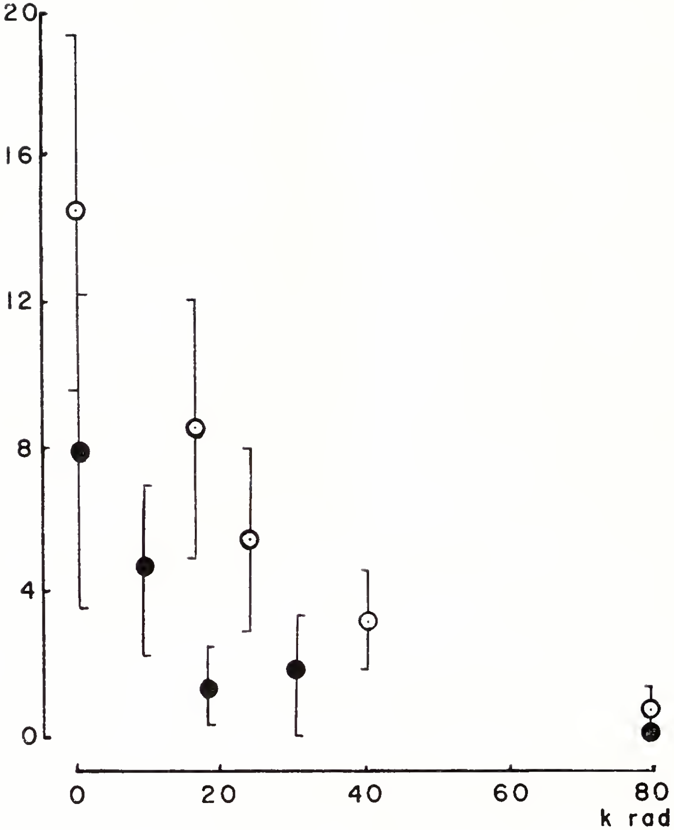


Figure 2. Average number of new hydranths added to colony starting material post-irradiation as a function of gamma-radiation dose. Open circles are 9° C colonies; closed circles are 18° C colonies. Vertical bars are one standard deviation above and below means.

In Figure 2, data concerning the number of new hydranth positions added to starters relative to the amount of gamma radiation received is presented. In both cases there is a decreasing number of hydranth positions produced with an increasing level of gamma radiation. The cause of the upward inflection of the 18° C-colony curve at 30 Krad is not known. In any event there is little to distinguish the average number of hydranths produced by starters which received 18 Krad and those that received 30 Krad of gamma radiation. In 18° C colonies, 80 Krad essentially stops hydranth production. The point at 80 Krad represents one new hydranth position generated by eight colonies in the five day period after irradiation.

Figure 3 presents data on the number of new uprights produced by new stolons after gamma radiation. There is a decrease in the number of new uprights produced with increasing dose of gamma radiation over the entire range from 0 through 80 Krad for the 9° C colonies; and a decrease in the number of new uprights produced with increasing dose over the range from 0 to 30 Krad for the

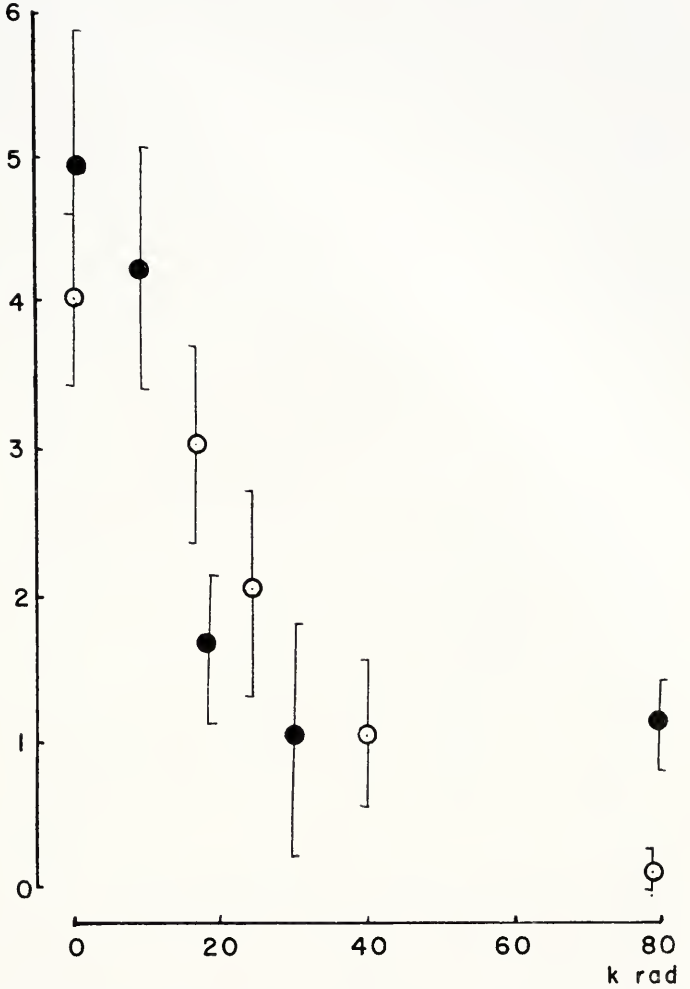


FIGURE 3. Average number of new uprights added to new stolons post-irradiation as a function of gamma-radiation dose in Krad. Open circles are 9° C colonies; closed circles are 18° C colonies.

18° C colonies, with little difference in the number of new uprights produced by 18° C colonies which had received 30 Krad, and those colonies which had received 80 Krad of gamma radiation. A possible explanation of this difference between 9° C and 18° C colonies will be presented in the discussion.

In Figure 4, data are presented on the number of hydranth positions added to uprights on stolons of 9° C and 18° C colonies receiving gamma radiation doses of 0 through 80 Krad. These curves are quite similar to those presented in Figure 3, there being a decrease in hydranth production with increasing radiation dose over the entire experimental range of 0 through 80 Krad for 9° C colonies; and a decrease in hydranth production with increasing radiation dose over the range

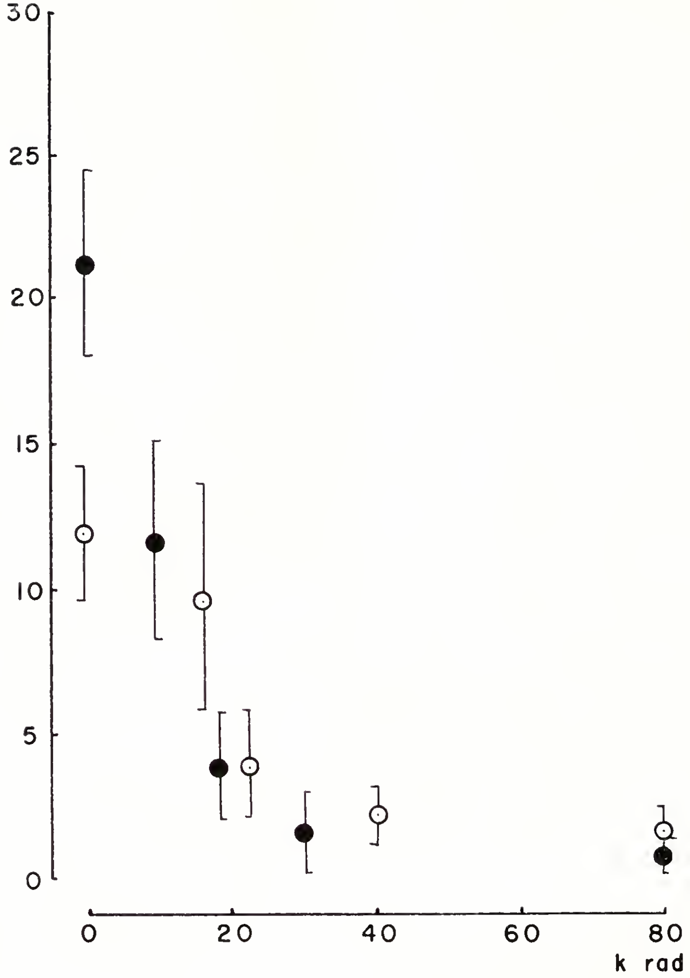


FIGURE 4. Average number of hydranths added to uprights of new stolons post-irradiation as a function of gamma-radiation dose in Krad. Open circles are 9° C colonies; closed circles are 18° C colonies. Vertical bars are one standard deviation above and below means.

from 0 through 80 Krad for 18° C colonies with little difference in hydranth production between 18° C colonies which received 30 Krad, and 18° C colonies which received 80 Krad of gamma radiation. Again, a possible explanation for the inflection in the curve of 18° C colonies for this growth function is presented in the discussion section of this paper.

Figures 5 and 6 present data on the average life span of new hydranths on new stolons (Fig. 5) of 9° C colonies which received from 0 through 80 Krad of gamma radiation; and on the average life span of hydranths on both new stolons and starters of 18° C colonies which received from 0 through 80 Krad of gamma radiation (Fig. 6). In all cases there is an increase in hydranth life span with

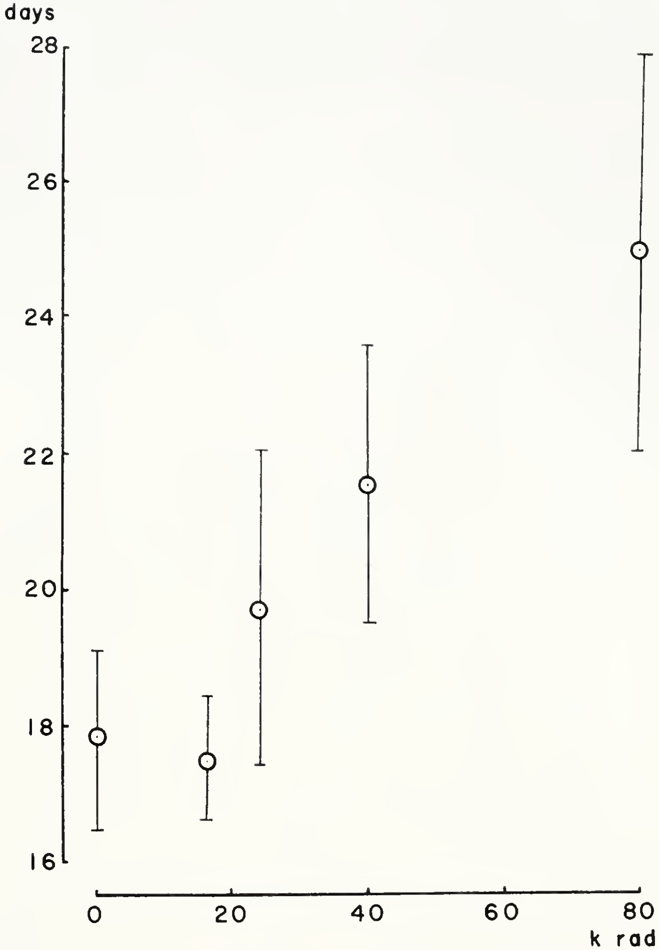


FIGURE 5. Average life span of new hydranths on new stolons of 9° C colonies as a function of gamma-radiation dose in Krad. Vertical bars are one standard deviation above and below means.

an increase in radiation dose. These data corroborate the data presented by Strehler (1964) and Brock and Strehler (1963) for x-irradiated hydranths of *Campanularia flexuosa*. While the curve is essentially linear over the range of 0 through 80 Krad for hydranths of 9° C colonies, there is an inflection in the curve of 18° C colonies at 30 Krad. There is little difference between the response of hydranths on starters and hydranths on stolons to gamma radiation over the range of 0 through 80 Krad in 18° C colonies.

DISCUSSION

Altman, Gerber, and Okada (1970) have stated that the primary radiation damage consists of damage to the cellular DNA itself. Employing this state-

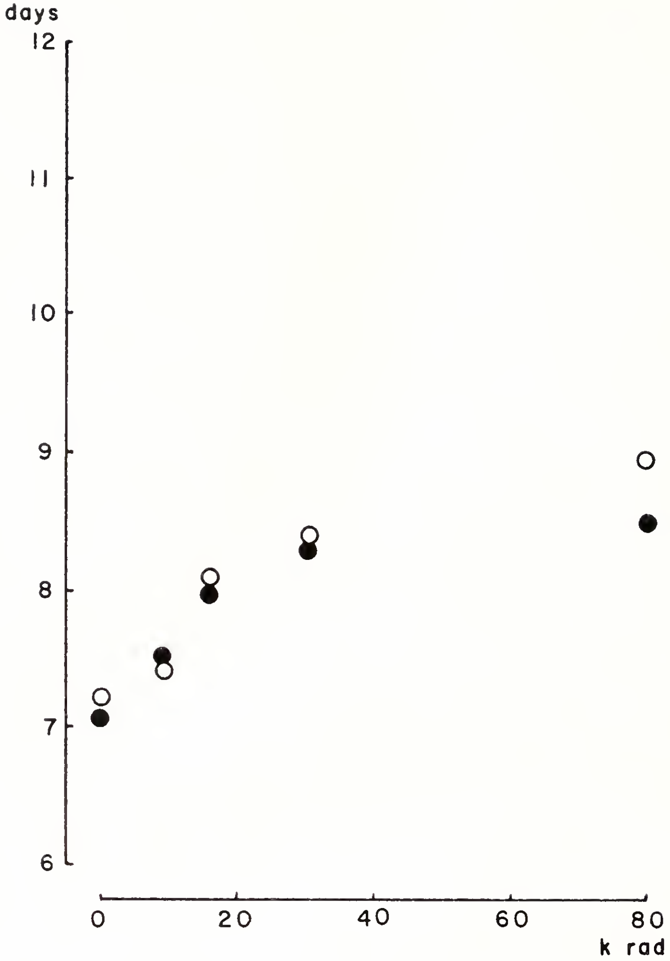


FIGURE 6. Average life span of hydranths on starters and new stolons of 18° C colonies as a function of gamma-radiation dose in Krad. Open circles are average life spans of hydranths on new stolons; closed circles are average life spans of hydranths on starters.

ment as a starting point, the data presented in the tables and figures are easily accounted for with only minor problems presented by the difference in temperature between the two experiments. If the primary radiation effect is damage to cellular DNA, and if the cellular DNA of *Campanularia flexuosa* functions as it does in other organisms, then it is not surprising to find that increasing doses of gamma radiation decreases the four growth functions of: (1) new stolon material produced, (2) new hydranth positions added to starters, (3) new uprights added to stolons, and (4) new hydranth positions added to uprights on stolons. All of these growth functions presumably depend upon the production of new cells. The greater the amount of radiation received by the organism, the greater is the chance that any particular portion of DNA will be disrupted by the ionizing radiation. Because of both aborted mitoses and the failure to manufacture proper messenger RNA molecules from the disrupted DNA, the production of new cells that would be involved in stolon growth (as stolon extension), hydranth production, and upright production would be less.

As a general rule, the higher the ambient temperature around colonies of *Campanularia flexuosa,* the greater is the rate of stolon growth, hydranth production, and upright production. Consequently any developmental period is shortened in time presumably including the time during which a particular structure is being determined. In slower growing colonies, *i.e.,* those grown at lower temperatures, there is a greater probability of a structure being covertly determined, but not yet morphologically distinct; that is, the period of covert determination is longer in slower-growing colonies than it is in faster-growing colonies. Ionizing radiation does not seem to interfere with subsequent differentiation after determination of that structure has occurred. If one applies this criterion to hydranth development, it would seem that hydranth development involves at least two separate determinations. At gamma radiation levels of 30 through 80 Krad, at least, new hydranth positions do appear but these new positions do not proceed past the pedicel stage of development except in rare cases. Those hydranths that have progressed to the cone stage of development at the time of irradiation proceed through the remaining developmental sequence, become normal-appearing feeding hydranths, and eventually regress, though they do not regenerate. Crowell (1974) reported a similar critical period of hydranth determination for dissociated hydranths, and actinomycin-D-treated hydranths of *Campanularia flexuosa,* as did Beloussov, Badenko, Katchurin, and Kurilo (1972) for both cyanide-treated and dinitrophenol-treated hydranths of this species.

Since in colonies grown at higher temperatures, the period of covert determination without morphological appearance is shorter than in colonies grown at lower temperatures, it would not be surprising to find, if the value of 30–40 Krad is accepted as the maximum dose needed to suppress determination, that a gamma radiation dose of 80 Krad would yield little or no greater effect in faster growing colonies with few structures in covert determination, but would cause an apparently greater effect in slower-growing colonies in which the period of covert determination is much longer. The lack of any effect on 18° C colonies dosed with 80 Krad over those dosed with 30 Krad will appear as an inflection at 30 Krad in the dose-response curves of those growth functions so considered. Likewise, the apparent effect in 9° C colonies at both 40 Krad and 80 Krad will be no inflection in these dose-response curves. It is suggested that if colonies were carefully controlled as to ambient temperature over the range of 9° C through 18° C in small steps of perhaps 1° C, and then if such colonies were gamma-irradiated at a dose level of 80 Krad, an estimate of the time required for the determination of hydranth positions could be made.

This work was performed while the first author was a National Cancer Institute Special Research Fellow. During his tenure, he was also a visiting assistant professor in the Department of Life Sciences, Indiana State University, Terre

Haute, Indiana. He wishes to thank Dr. Charles D. Barnes of that department for his aid and sponsorship. This work was performed under fellowship 1 FO3 C14 53590–01 from the National Institutes of Health.

SUMMARY

1. Colonies of *Campanularia flexuosa* were gamma-irradiated with doses ranging from 0 through 80 Krad. Several growth functions were monitored. These growth functions include hydranth production by starters and stolons, upright production by stolons, stolon growth, and longevity of hydranths on both starters and stolons.

2. All growth functions show a decrease with increasing doses of gamma radiation, except hydranth longevity. Hydranth longevity increases with increasing dose.

3. A difference in temperature yields differences in the growth-response curves of these growth functions.

4. The implications of the dose-response curves are discussed in terms of the hypothesis that the primary effect of ionizing radiation is damage to DNA molecules.

LITERATURE CITED

ALTMAN, K. I., G. GERBER AND S. OKADA, 1970. *Radiation Biochemistry.* Volume I: Cells. Academic Press, New York and London, 366 pp.

BELOUSSOV, L. V., L. A. BADENKO, A. L. KATCHURIN AND L. F. KURILO, 1972. Cell movements in the morphogenesis of hydroid polyps. *J. Embryol. Exp. Morphol.,* **27**: 317–337.

BROCK, M. A., AND B. L. STREHLER, 1963. Studies on the comparative physiology of aging. IV. Age and mortality of some marine cnidaria in the laboratory. *J. Gerontol.,* **18**: 23–28.

CROWELL, P. S., 1953. The regression-replacement cycles of hydranths of *Obelia* and *Campanularia. Physiol. Zool.,* **25**: 319–327.

CROWELL, S., 1974. Morphologenetic events associated with stolon elongation in colonial hydroids. *Amer. Zool.,* **14**: 665–671.

DANIEL, G. E., AND H. D. PARK, 1951. The effects of x-ray treated media on Hydra tentacles. *J. Cell. Comp. Physiol.,* **38**: 417–425.

DANIEL, G. E., AND H. D. PARK, 1953. Glutathione and x-ray injury in Hydra and Paramecium. *J. Cell. Comp. Physiol.,* **43**: 359–368.

MCLAUGHLIN, W. L., E. K. HUSSMANN, H. H. EISENLOHR AND L. CHALKLEY, 1971. A chemical dosimeter for monitoring gamma-radiation doses of 1–100 Krad. *Int. J. Appl. Rad. Isot.,* **22**: 135–140.

PUCKETT, W. O., 1935. The effects of x-irradiation on the regeneration of the hydroid *Pennaria tiarella. Anat. Rec.,* **64**: 30.

STREHLER, B. L., 1964. Studies on the comparative physiology of aging. III. Effects of x-irradiation dosage on age-specific mortality rates of *Drosophila melanogaster* and *Campanularia flexuosa. J. Gerontol.,* **19**: 83–87.

WERMUTH, J. F., AND C. D. BARNES, 1967. The effects of x-irradiation on stolon growth in *Campanularia flexuosa. J. Gen. Physiol.,* **50**: 2505.

WERMUTH, J. F., AND C. D. BARNES, 1968. Differential glutathione protection of radiation effects at two different sites in *Campanularia flexuosa. Radiat. Res.,* **35**: 496.

WERMUTH, J. F., AND C. D. BARNES, 1969. Differential radioprotection by glutathione of two growth functions in the hydroid *Campanularia flexuosa. Biol. Bull.,* **137**: 375–383.

Continued from Cover Two

4. ***Literature Cited.*** The list of references should be headed LITERATURE CITED, should conform in punctuation and arrangement to the style of recent issues of The Biological Bulletin, and must be typed *double-spaced* on separate pages. Note that citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by Biological Abstracts and Chemical Abstracts, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by Biological Abstracts of those abstracted (most recent issue: November, 1972). Foreign authors, and others who are accustomed to use The World List of Scientific Periodicals, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. at £0.65 or $1.75) useful, since it sets out the World List abbreviations for most biological journals with notes of the USASI abbreviations where these differ. Chemical Abstracts publishes quarterly supplements of additional abbreviations. The following points of reference style for The Biological Bulletin differ from USASI (or modified World List) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in World List *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (not strictly as USASI usage, *i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.* not *J.Cell.Comp.Physiol.*)

E. We strongly recommend that more unusual words in journal titles be spelled out in full, rather than employing lengthy, peculiar "abbreviations" or new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation. Even in more familiar languages, *Z. Vererbungslehre* is preferred to *Z. VererbLehre* (World List) or *Z. Vererbungsl.* (USASI). *Accurate and complete communication of the reference is more important than minor savings in printing costs.*

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some Biological Abstracts listings).

H. Spell out *London, Tokyo, Paris, Edinburgh, Lisbon, etc.* where part of journal title.

I. Series letters *etc.* immediately before volume number.

J. A few well-known international journals in their preferred forms rather than World List or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond.; Science, N.Y.; Evolution, Lancaster, Pa.*)

K. The correct abbreviation for The Biological Bulletin is *Biol. Bull.*

5. ***Figures.*** The dimensions of the printed page, 5 by $7\frac{3}{8}$ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction. Explanatory matter should be included separately in legends as far as possible, although the axes should always be numbered and identified on the illustration itself. Figures should be prepared for reproduction as either line-cuts or halftones; no other methods will be used. Figures to be reproduced as line-cuts should be drawn in black ink on white paper, good quality tracing cloth or plastic, or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on board, and both designating numbers or letters and scale-bars should be affixed directly on the figures. We recommend that halftones submitted to us be mounted prints made at about $1\frac{1}{2}$ times the linear dimensions of the final printing desired (the actual best reductions are achieved from copy in the range from $1\frac{1}{4}$ to 2 times the linear dimensions). As regards line-blocks, originals can be designed for even greater reductions but are best in the range $1\frac{1}{2}$ to 3 times. *All* figures should be numbered in consecutive order, with no distinction between text and plate-figures. The author's name should appear on the reverse side of all figures, and the inked originals for line-blocks must be submitted for block-making.

6. ***Mailing.*** Manuscripts should be packed flat. All illustrations larger than $8\frac{1}{2}$ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.

Reprints. Reprints may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

CONTENTS

Blanquet, Richard S. and Bruce Wetzel
Surface ultrastructure of the Scyphopolyp, *Chrysaora quinquecirrha* 181

Daniels, Bruce A. and Roy T. Sawyer
The biology of the leech *Myzobdella lugubris* infesting blue crabs and catfish 193

Dunn, Daphne Fautin
Reproduction of the externally brooding sea anemone *Epiactis prolifera* Verrill, 1869 199

Holland, Nicholas D., John C. Grimmer and Hiroshi Kubota
Gonadal development during the annual reproductive cycle of *Comanthus japonica* (Echinodermata: Crinoidea) 219

Keck, Richard T., Don Maurer and Henry Lind
A comparative study of the hard clam gonad developmental cycle .. 243

Kunkel, J. G.
Cockroach molting. I. Temporal organization of events during the molting cycle of *Blattella germanica* (L.) 259

Milkman, Roger
Specific death sites in a *Drosophila* population cage 274

Ravindranath, M. H.
Effects of temperature on the morphology of hemocytes and coagulation process in the mole-crab *Emerita* (=*Hippa*) *asiatica* 286

Robertson, James D.
Osmotic constituents of the blood plasma and parietal muscle of *Squalus acanthias* L. 303

Sláma, Karel and Magdalena Hodková
Insect hormones and bioanalogues: their effect on respiratory metabolism in *Dermestes vulpinus* L. (Coleoptera) 320

Sulkin, Stephen D.
The influence of light in the depth regulation of crab larvae 333

Wermuth, Jerome F. and Charles D. Barnes
Dose-response effects of gamma-radiation on several growth functions of *Campanularia flexuosa* 344

Volume 148 Number 3

end

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

JOHN M. ANDERSON, Cornell University
JOHN B. BUCK, National Institutes of Health
JOHN O. CORLISS, University of Maryland
JOHN D. COSTLOW, Duke University
CATHERINE HENLEY, University of North Carolina
GEORGE O. MACKIE, University of Victoria
F. H. RUDDLE, Yale University
BERTA SCHARRER, Albert Einstein College of Medicine
HOWARD A. SCHNEIDERMAN, University of California, Irvine
GROVER C. STEPHENS, University of California, Irvine
CARROLL M. WILLIAMS, Harvard University
EDWARD O. WILSON, Harvard University

W. D. RUSSELL-HUNTER, Syracuse University
Managing Editor

JUNE, 1975

b

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to THE BIOLOGICAL BULLETIN, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers, $7.00. Subscription per volume (three issues), $18.00, (this is $36.00 per year for six issues).

Communications relative to manuscripts should be sent to Dr. W. D. Russell-Hunter, Marine Biological Laboratory, Woods Hole, Massachusetts 02543 between May 23 and September 1, and to Dr. W. D. Russell-Hunter, P.O. Box 103, University Station, Syracuse, New York 13210, during the remainder of the year.

Copyright © 1975, by the Marine Biological Laboratory

Second-class-postage paid at Lancaster, Pa.

INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, footnotes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

Continued on Cover Three

Vol. 148, No. 3 June, 1975

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

NATURALLY-OCCURRING HEMAGGLUTININ IN A TUNICATE *HALOCYNTHIA PYRIFORMIS*

Reference: *Biol Bull.,* **148**: 357–369. (June, 1975)

ROBERT S. ANDERSON AND ROBERT A. GOOD

Sloan-Kettering Institute for Cancer Research,
Walker Laboratory,
145 Boston Post Road, Rye, New York 10580

Naturally-occurring hemagglutinating proteins (lectins) are widely distributed in nature and are finding numerous applications in immunologic and cancer research. The most thoroughly studied lectins are those derived from plants such as concanavalin A (from the jack bean), red kidney bean agglutinin (PHA), wheat germ agglutinin, and soybean agglutinin. Phytohemagglutinins possess many interesting biological and chemical properties in addition to their ability to agglutinate red cells. Some lectins are specific for human ABO and MN blood groups and have been used in blood typing and studies of blood group specificity (Boyd, 1963; 1970). Certain lectins act as mitogens stimulating blast formation and mitosis of lymphocytes (Robbins, 1964; Naspitz and Richter, 1968). Since lectins bind specifically to cell-surface saccharides, they provide a new method for the study of the architecture of cell surfaces. Lectins are of particular interest because some are capable of preferential agglutination of cultured mammalian cells that have been transformed by oncogenic viruses or chemical oncogens, as well as cells from spontaneous tumors (Aub, Sanford and Cote, 1965; Burger, 1969; Inbar and Sachs, 1969; Sela, Lis, Sharon and Sachs, 1970).

The presence of these unusual biochemical properties in plant lectins has stimulated renewed interest in naturally-occurring hemagglutinins from other sources, particularly invertebrate animals. In the course of our study of humoral immune factors of tunicates, we found a quite active panagglutinin in *Halocynthia pyriformis,* which will be described in this paper. Tunicates occupy a unique phylogenetic niche between the vertebrates and invertebrates. In light of the great differences in immunological specificity and memory phenomena between vertebrate and invertebrates, tunicates assume a high degree of importance in the study of the phylogeny of immunity. Recently several studies of hemagglutinins in tunicates have appeared (Fuke and Sugai, 1972; Wright, 1974); the lectin described in this paper apparently differs in both biochemistry and activity from

Copyright © 1975, by the Marine Biological Laboratory
Library of Congress Card No. A38–518

hemagglutinins from other tunicate species. In addition to basic biophysical and chemical characteristics of the lectin, we present data on specificity, inhibition by saccharides, and sensitivity to chemically-modified erythrocytes.

Materials and Methods

Experimental animals and hemolymph collection

The ascidians, *Halocynthia pyriformis* and *Boltenia ovifera,* were collected in the Bay of Fundy by Marine Research Associates, New Brunswick, Canada; *Ciona intestinalis* was supplied by the Marine Biological Laboratory, Woods Hole, Massachusetts. The animals were held at approximately 15° C in 150 gallon marine aquaria (Aquarium Systems, Inc., Eastlake, Ohio) until used. After expressing the sea water held within the branchial sac, the ascidians were bled through incisions made in the tunic beneath the excurrent siphon. As much as 10 ml of hemolymph could be collected from one large ascidian. The hemolymph was collected on ice and used immediately after removal of hemocytes by centrifugation.

Hemagglutination assays

Pooled normal blood in Alsever's solution (1:1) from various mammalian and avian species were obtained from the Animal Blood Centre, Inc. (Syracuse, New York). The cells were washed in Alsever's solution and resuspended to give stock solutions of 10^9 cells/ml. The stock solutions were stored at 4° C. Prior to use, aliquots of the stock erythrocytes were diluted in 0.15 M NaCl to a concentration of 10^8/ml. Hemolymph (cell-free) was serially diluted in 0.15 M NaCl and an equal volume of dilute erythrocytes was added to each tube. Agglutination titers are expressed as the reciprocal of the lowest dilution of hemolymph which caused visible agglutination at a given time of incubation.

Hemolymph treatments

The effect of trypsin was determined by incubating tunicate hemolymph for 60 min at 37° C, in the presence of 5–500 μg/ml Type III trypsin. All chemicals, except where otherwise indicated, were obtained from Sigma Chemical Company, St. Louis, Mo.

Hemolymph was heated in a thermostatically-controlled water bath over a wide range of temperatures and for various time intervals, to determine the heat lability of the hemagglutinin. Hemagglutination was carried out at various pH values in appropriate buffers to determine its sensitivity to hydrogen ion concentration.

Rapid freezing and thawing of hemolymph was carried out by freezing in an ethanol-dry ice mixture followed by thawing under warm water.

Several methods were used to remove most of the divalent cations from hemolymph samples. The hemolymph was dialyzed at room temperature against Ca^{++} and Mg^{++}-free Hank's balanced salt solution (Grand Island Biological Company, New York) containing 0.02% EDTA (ethylenediamine tetraacetic acid, disodium salt). Hemolymph and EDTA (1.56×10^{-3}–2.5×10^{-2} M final concentration) were incubated for 3 hr at room temperature followed by dialysis against 0.15 M NaCl, 60 hr at room temperature. The calcium and/or magnesium ion concentration in dialyzed or untreated hemolymph was adjusted by additions of $CaCl_2$ or $MgCl_2$.

Dialysis of hemolymph for 24 hr at room temperature against deionized water resulted in the production of a light flocculation. This flocculant material was removed by centrifugation at about 1090 g for 10 min and was readily soluble in 0.15 M NaCl. This fraction was shown to contain almost all of the hemagglutinating activity of native hemolymph. The hemagglutinin could also be obtained by treating the hemolymph with 30–50% ammonium sulfate (18 hr at 4° C). The resulting precipitate was centrifuged out as above, washed several times with deionized water, and dissolved in 0.15 M NaCl. Total hemagglutinating activity was recovered in the precipitate; the supernatant had no activity.

Adsorption studies were carried out to determine the specificity of the hemagglutinin. Hemolymph was adsorbed with 10^9 washed erythrocytes/ml hemolymph for 1 hr at 37° C. The red cells were removed by centrifugation, and the hemolymph was again adsorbed with a comparable quantity of fresh erythrocytes at the same temperature and time. The red cells were again centrifuged out and the hemolymph serially diluted for the agglutination assay. Control hemolymph was unadsorbed but was incubated at 37° C for 2 hr.

Since many plant and invertebrate lectins combine with specific sugar moieties, the inhibitory action of certain monosaccharides commonly found in the glycoproteins of red cell membranes on the tunicate hemagglutinin was assayed. The sugars tested included D-glucose, D-galactose, D-mannose, L-fucose, L-arabinose, D-xylose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, and N-acetylneuraminic (sialic) acid. Hemolymph was serially diluted in 10^{-5}–0.25×10^{-3} M solutions of the various sugars, and the mixture was incubated 1 hr at 23° C. Horse erythrocytes were then added, as previously described, and the activity of the hemagglutinin determined. When the indicator cells used were human red cells, the agglutination studies were carried out in the presence of 20 mM $CaCl_2$.

Chemical modification of erythrocytes

Enzyme-treated erythrocytes have been shown to be agglutinated more strongly by plant lectins than untreated cells. The effect of tunicate hemagglutinin on several kinds of chemically-altered cells is reported in this paper.

Red cells (10^8/ml) were incubated in an equal volume of 0.1 mg Sigma Type III (bovine pancreatic) trypsin/ml Hank's balanced salt solution for 10–30 min at 37° C. The cells were then washed 4 times in saline and resuspended so that the final red cell concentration was 10^8/ml. Horse erythrocytes (2 ml of 10^9/ml) were incubated in 20 ml 0.01% bromelain for 10–30 min at 37° C, followed by 4 washes and resuspended in saline. The agglutination of erythrocytes (1 volume packed red cells: 1 volume enzyme solution) incubated at 37° C for 10–60 min in the presence of Sigma Type V protease ("pronase" from *Streptomyces griseus*) was also studied.

RESULTS

Hemagglutinating activity in Halocynthia pyriformis *hemolymph*

Halocynthia hemolymph contains naturally-occurring hemagglutinin(s) for a wide range of avian and mammalian erythrocytes. The agglutinin is specific for certain blood cells; its titer for any given kind of erythrocyte shows little variation among individual ascidians of the same species. The red cells could be divided into two major groups with regard to sensitivity to the lectin. Weakly agglutinated erythrocytes (titers ranging from 2 to 32) include human, pigeon, rabbit, sheep, swine, goat, calf, and ox. Much higher titers (64–512) were consistently recorded for duck, goose, chicken, turkey, guinea pig, and horse red cells. Horse cells were the most readily agglutinated and were frequently used as indicator cells in further studies of the properties of the hemagglutinin. As discussed elsewhere, the presence of Ca^{++} in the median generally increased the titers for all red cell types. The hemagglutinin was not specific as to the A, B, O antigens of human erythrocytes.

Homogenates of branchial sac tissue also contained agglutinating activity for human and horse erythrocytes. It is quite possible that this activity resulted from the presence of hemolymph in this tissue; it was technically impossible to obtain hemolymph-free tissue samples.

The hemagglutinating activity of the hemolymph was not affected by incubation with low concentrations of trypsin for 60 min at 37° C. However, trypsin concentrations over 500 μg/ml hemolymph did substantially reduce hemagglutination.

Sensitivity of the lectin to heat, pH and freezing and thawing and storage

The lectin was shown to be heat-labile at temperatures exceeding 50° C. Incubation of hemolymph at temperatures ranging from 1°–48° C, for periods of 1–2 hr, did not inhibit its strong hemagglutinating activity for horse erythrocytes. This activity was abolished by any of the following time and temperature regimens: 120 min at 50°, 60 min at 53°, 30 min at 55°, or 15 min at 58° C.

The agglutination of horse erythrocytes by tunicate hemolymph was slight at extreme pH values (5 and 11). Activity was quite strong over a comparatively wide range of pH 6–10. The highest titers were recorded about pH 8–9.

Repeated (20 ×) freezing and thawing of the hemolymph had no appreciable effect on the titer of the lectin against horse red cells.

The hemagglutinin retained full activity for at least 2 days when held at 25° C. No activity was lost during the first 5 days at 4° C; thereafter the activity gradually declined but was still quite strong for as long as 2 months at this temperature. Stability could be considerably prolonged by storing the hemolymph at −5° C.

Role of divalent cations in hemagglutinin activity

Dialysis of hemolymph against EDTA-containing balanced salt solution for 24 hr resulted in marked diminution of the ability of the hemolymph to agglutinate horse or human B erythrocytes. Hemolymph incubated with 6.25×10^{-3} M (or higher) EDTA for 3 hr at room temperature lost its activity for human A, B, or horse erythrocytes; the EDTA was removed by dialysis prior to determination of titers.

If the hemagglutination reaction was carried out in the absence of added Ca^{++}, no agglutination of human A red cells was detected; the presence of 20 mM $CaCl_2$ resulted in marked hemagglutination with titers of 128 or higher. Horse, swine, and calf erythrocytes agglutinated in the absence of added Ca^{++} but, with the exception of calf cells, were more extensively agglutinated in the presence of 20 mM $CaCl_2$. This stimulatory effect of Ca^{++} could be shown in concentrations of 1–100 mM added to untreated hemolymph. The effects of low concentrations of Ca^{++} on hemagglutinin activity are more pronounced if the hemolymph is dialyzed against water (24 hr, 25° C) and the flocculated protein redissolved in saline. The addition of 5 mM of $CaCl_2$ quadruples the titer observed in the absence of Ca^{++}; further increase in Ca^{++} concentration has no effect on the titer.

It would appear that Mg^{++} cannot substitute for Ca^{++} in these reactions. At low concentrations (0.1–1 mM) added Mg^{++} has little or no effect on hemagglutination titers; however, at higher concentrations (10–100 mM) Mg^{++} in the medium inhibits the reaction. The hemagglutinating activity of protein derived from hemolymph by dialysis against deionized water is markedly inhibited by 5 mM $MgCl_2$ and abolished by 30 mM Mg^{++} present in the medium.

Effect of temperature of incubation

The effect of incubation temperature on the hemagglutinin titer for human A, horse, sheep, rabbit, guinea pig, ox, and goat erythrocytes was determined. Generally, the titers obtained at 1° C were about the same as those measured at 25° C for all erythrocytes tested; however, the reactions were less intense at the lower temperature. Stronger reactions and higher titers were usually recorded at 37° C, particularly in the case of sheep, guinea pig, and goat erythrocytes.

Dialysis and salting out of hemagglutinin

The hemagglutinin is resistant to dialysis against 0.15 M NaCl for 24 hr at room temperature, provided Ca^{++} is present during hemagglutination, particularly in the case of human erythrocytes. If the hemolymph is dialyzed against deionized water, a light flocculation takes place; by 48 hr no additional material will come out of solution. The material was shown to represent almost the total serum protein (on the basis of Lowry protein determinations) and could be easily centrifuged out of suspension and redissolved in 0.15 M NaCl. The hemagglutinating activity resides only in this protein fraction; the soluble material remaining after dialysis against deionized water is devoid of activity.

It was also found that the hemagglutinin could be rendered insoluble by treatment of the hemolymph with 30% or 50% ammonium sulfate for 18 hr at 4° C. This precipitate can be washed in deionized water and dissolved in 0.15 M NaCl with restoration of agglutinating activity.

Adsorption studies

The specificity of the natural hemagglutinin(s) in *Halocynthia pyriformis* hemolymph was tested by adsorption studies using a number of mammalian eryth-

TABLE I

Adsorption studies of Halocynthia pyriformis *lectin*

Test erythrocytes	Hemagglutination titer after adsorption with erythrocytes of:				
	Unadsorbed	Horse	Human A*	Goat	Calf
Horse	256	32	256	32	32
Swine	32	0	4	†	0
Human A	32	2	32	0	2
Calf	32	0	2	†	0
Goat	16	0	4	0	†
Ox	4	0	2	0	†
Sheep	8	0	4	0	†
Rabbit	4	0	4	0	†

* In the presence of 20 mM $CaCl_2$.
† Not done.

rocytes (Table I). Horse red cells are strongly agglutinated by *Halocynthia* hemolymph; this activity is unaffected by adsorption with human A cells, but can be substantially reduced by adsorption with horse, goat, or calf erythrocytes. Adsorption of hemolymph with horse, goat, or calf red cells also markedly reduces or eliminates hemagglutinating activity against swine, human A, calf, goat, ox, sheep, and rabbit cells in all instances tested. Hemolymph adsorbed with human A erythrocytes retains its ability to agglutinate these cells and all other mammalian red cells tested. The data indicate that, particularly with the nonhuman mammalian erythrocytes, adsorption with any given red cells will not only decrease or abolish activity of the agglutinin for the cells in question, but will also alter the hemagglutination of many other kinds of erythrocytes.

Agglutination of chemically-modified erythrocytes

Bromelain-treated horse erythrocytes were agglutinated to the same extent as untreated cells. However, both trypsinized and protease-treated mammalian red cells were agglutinated more strongly than untreated cells (Table II). Human A erythrocytes, which are relatively refractory to the action of the lectin in the absence of Ca^{++}, were strongly agglutinated in the absence of Ca^{++} after only 10 min incubation in a 0.1 mg/ml trypsin solution. Similar marked increases in titer of the agglutinin for human B and O, ox, sheep, horse, and calf erythrocytes could be produced by 10 or 30 min incubations in trypsin. Treatment of horse, calf, or swine red cells with protease (0.1 mg/ml) caused progressive increases in hemagglutinin titers with increasing time of incubation from 10 to 60 min.

Another ascidian, *Boltenia ovifera*, was shown to possess a lectin which reacts weakly with a variety of mammalian erythrocytes. The titer of this lectin was no greater for trypsinized sheep cells than for untreated sheep red cells. However, trypsinization did increase agglutinin titers for horse and human erythrocytes.

Inhibition of hemagglutinin by sugars

Ascidian hemolymph was reacted with a number of monosaccharides which are found in glycoproteins in order to give information concerning the nature of receptor sites of the lectin. The sugars used in this study included D-glucose, D-galactose, D-mannose, L-fucose, L-arabinose, D-xylose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, and N-acetyl-neuraminic (sialic) acid. None of these sugars exerted inhibitory activity against the lectin at concentrations of 10^{-5} to 10^{-4} M. However, incubation of hemolymph with 0.25×10^{-3} M sialic acid markedly inhibited the agglutination of both horse and human A erythrocytes; the other sugars were not inhibitory at this concentration.

Hemagglutinins in several other ascidians

Boltenia ovifera, like *Halocynthia pyriformis,* is a large ascidian which is common on subtidal ledges in the Bay of Fundy. However, the hemolymph of *Boltenia* lacks a powerful hemagglutinin for either avian or mammalian red cells. Calf, duck, goat, and guinea pig erythrocytes were not agglutinated by *Boltenia* hemolymph; titers of 2–4 were obtained using sheep, goose, chicken, turkey, pigeon, ox, horse, and human red cells.

Ciona intestinalis is probably one of the most frequently studied ascidians. We bled a number of these animals and found a low level of hemagglutinating activity in their hemolymph. After 2 hr of incubation, titers of 2–4 were recorded for ox, goat, and human B and O cells; the titer for human A and horse cells was somewhat higher (about 16). Sheep and guinea pig erythrocytes were not agglutinated.

DISCUSSION

The presence of hemagglutinins in the hemolymph of invertebrates has been known since at least 1903 (Noguchi, 1903). Tyler (1946) reported the presence of heteroagglutinins in the hemolymph of a number of invertebrates. A resurgence of interest in these factors has taken place recently, largely as a result of their implication as a part of a system for the recognition of foreign antigens (Boyden,

TABLE II

Agglutination of enzyme-treated erythrocytes

Treatment*	Hemagglutinin titer				
	Erythrocyte type	Time of incubation with enzyme			
		0′	10′	30′	60′
Pronase	Swine	16	64	64	128
	Horse	128	128	256	512
	Calf	8	16	16	32
Trypsin	Human A	4	64	128	†
	Human B	32	†	64	†
	Human O	16	†	64	†
	Horse	256	†	1024	†
	Sheep	4	†	32	†
	Ox	4	64	512	†

* 1 vol. 0.1 mg enzyme/ml saline: 1 vol. packed erythrocytes, 37° C.

† Not done.

1966; Tripp, 1966; Cushing, 1967) and because of the unusual properties of plant lectins already mentioned. Recently natural hemagglutinins have been reported in numerous invertebrates including sponges (Gold, Phelps, Khalap and Balding, 1974), pelecypod mollusks (Jenkin and Rowley, 1970; Acton, Bennett, Evans and Schrohenloher, 1969; Cornick and Stewart, 1973), gastropod mollusks (Pemberton, 1970a; 1970b; Bizot, 1971; Hammarström and Kabat, 1971; Matsubara and Boyd, 1974; Pauley, Granger and Krassner, 1971); annelids (Cooper, Lemmi and Moore, 1974); arthropods (Miller, Ballback, Pauley and Krassner, 1972; Marchalonis and Edelman, 1968; Finstad, Litman, Finstad and Good, 1972; Pauley, 1973; Scott, 1971; Anderson, Day and Good, 1972), and echinoderms (Finstad, Litman, Finstad and Good, 1972).

Halocynthia hemagglutinin was assayed in hemolymph cleared of blood cells by centrifugation. The source of this lectin is not known at the present time. The possibility of its being released by hemocytes *in vivo* cannot be ruled out. However, it is unlikely that this factor was released by hemocyte lysis during hemolymph preparation. Extracts of osmotically-lysed blood cells had no hemagglutinating activity even after concentration by ultrafiltration.

Halocynthia pyriformis hemolymph contains naturally-occurring hemagglutinin(s) against a wide range of avian and mammalian red cells. The activity was directed against the erythrocytes of all (14) species tested and was characterized by relatively high titers (64–512) for the red cells of 6 species. The hemagglutinin of the tunicate *Ciona intestinalis* is quite weak in comparison, with reactivity against many red cell types but with titers never exceeding eight (Wright, 1973; 1974). The natural lectins reported in several other tunicates (*Styela plicata* and *Halocynthia hilgendorfi*) are stronger and show more specificity (Fuke and Sugai, 1972). These agglutinins were directed against the erythrocytes of rats, rabbits, and mice but were inactive against fish, frog, snake, sheep, and guinea pig red cells.

Human blood group specificity, which is marked in albumin gland hemagglutinins from certain gastropod mollusks (Prokop, Uhlenbruck, Rothe and Cohen, 1974), is lacking in *H. pyriformis*. This lack of specificity has been reported in other urochordates (Wright, 1974) and is typical of most hemolymph lectins (Brown, Almodovar, Bhatia and Boyd, 1968; Tripp, 1966; Hall and Rowlands, 1974b).

A lack of sensitivity of invertebrate lectins to proteolytic enzymes has been reported for a number of species (Uhlenbruck, Reifenberg and Prokop, 1971; Pauley, 1974; Fuke and Sugai, 1972; Ryoyama, 1974). We report that *H. pyriformis* lectin is inactivated by certain trypsin concentrations as is the case for lobster agglutinin (Hall and Rowlands, 1974a). The protein nature of this tunicate lectin was indicated in several ways. Treatment of the hemolymph with trichloroacetic acid precipitated essentially all of the proteins present, protein-free hemolymph was lacking in hemagglutinating activity. Most of the hemolymph protein could be removed by ultrafiltration; the total activity of the lectin resided in this material and hemagglutination titers were proportional to protein concentrations over the dilution range tested.

H. pyriformis hemagglutinin begins to show heat lability at about 50° C and can be totally inactivated by incubating 15 min at 58° C. Wright (1974) reports that *Ciona* lectin is heat labile at temperatures exceeding 70° C. However, *Styela plicata* coelomic fluid retains full activity even after 30 min at 100° C (Fuke and Sugai, 1972). The lectins of most invertebrates so far studied, such as arachnids, crustaceans, mollusks and insects, are heat labile in the range 50°–70° C (Finstad, Litman, Finstad and Good, 1972; Hall and Rowlands, 1974a; Tripp, 1966; Johnson, 1964; Cornick and Stewart, 1973; Scott, 1971; Pauley, 1974). The hemagglutinin of the sea urchin *Hemicentrotus pulcherrimus* is not affected by temperatures of 100° C; however, the lectins of other sea urchnis are heat labile from 70°–85° C (Ryoyama, 1974).

H. pyriformis lectin is active over a wide pH range (6–10) and is most active around pH 8–9. Fuke and Sugai (1972) report essentially the same properties for hemagglutinin from another tunicate species. The hemagglutinins of the lobster, sea hare, crayfish, sea urchin and oyster are also active over broad ranges of pH values (Hall and Rowlands, 1974a; Pauley, 1974; Ryoyama, 1974; Cornick and Stewart, 1973).

We report that tunicate hemagglutinin does not lose activity after repeated freezing and thawing. This is also a property of the lectins of the sea hare, crayfish and blue crab (Pauley, 1974); these agglutinins could be stored with full activity for at least 6 months at −12 or −25° C. *H. pyriformis* hemagglutinin is fully active for at least several months at −5° C and loses little activity when stored at 4° C. *Ciona* lectin responded to storage in a similar fashion (Wright, 1973).

Ca^{++} is required for the expression of biological activity of many naturally-occurring invertebrate hemagglutinins, such as those from *Limulus* (Finstad, Litman, Finstad and Good, 1972; Marchalonis and Edelman, 1968); lobster (Hall and Rowlands, 1974a); oyster (Acton, Bennett, Evans and Schrohenloher, 1969), and tunicate (Wright, 1973). A similar Ca^{++} dependency exists for *H. pyriformis* lectin based on its inactivation with EDTA and the augmented titers observed in the presence of added Ca^{++}. McDade and Tripp (1967) found that oyster hemagglutinin was active against sheep and rabbit erythrocytes in the absence of Ca^{++}, but did not agglutinate human red cells without Ca^{++} in the medium. McDade and Tripp (1967) also observed that Ca^{++} was required for heat stability of the lectin; this was confirmed by Cornick and Stewart (1973). Ca^{++} enhanced the activity of sea urchin lectin, whereas Mg^{++} was ineffective (Ryoyama, 1974); similar results were reported in the case of *Limulus* (Marchalonis and Edelman, 1968). The inability of Mg^{++} to substitute for Ca^{++} in lectin activation is demonstrated in the tunicate *H. pyriformis*. However, Wright (1973) reports that both Ca^{++} and Mg^{++} enhance the hemagglutinin titer in *Ciona* and both ions will restore the activity of EDTA-treated hemolymph. It has been reported that Ca^{++} is not required for activity of *Styela plicata*, sea hare or blue crab hemagglutinin (Fuke and Sugai, 1972; Pauley, 1974).

We report that *H. pyriformis* lectin loses no activity after dialysis against 0.15 M NaCl for 24 hr. If the agglutinin is dialyzed against deionized water for a similar period, a light flocculation occurs. This material can be centrifuged out of suspension, is quite soluble in 0.15 M NaCl, and can be shown to be a protein by the Lowry and biuret methods. The total hemagglutinating activity of the hemolymph can be recovered in this fraction. The natural hemagglutinins of other tunicates are also stable after dialysis *vs.* saline (Fuke and Sugai, 1972; Wright, 1973). Most invertebrate lectins are nondialyzable and their activity will precipitate out with the serum proteins when dialyzed against distilled water (McDade and Tripp, 1967; Scott, 1971)

Adsorption studies of *H. pyriformis* hemagglutinin indicate a considerable degree of cross-reactivity. Similar results were obtained by Wright (1973) using *Ciona* and Fuke and Sugai (1972) using several other ascidian species. This general lack of specificity of hemagglutinins has been reported for most invertebrate species. In arthropods and mollusks adsorption with erythrocytes from a given species usually reduces lectin titers against that cell, as well as affecting the activity against other types of red cells (Tripp, 1966; Scott, 1971; Hall and Rowlands, 1974b).

The agglutination of enzyme-treated erythrocytes by tunicate lectin has not been previously reported. Incubation of red cells with either trypsin or pronase greatly increased their susceptibility to the hemagglutinin of *H. pyriformis.* Untreated human erythrocytes are weakly agglutinated in the absence of Ca^{++}; however, the same cells when trypsinized agglutinate strongly regardless of Ca^{++} concentration. Hall and Rowlands (1974b) reported that trypsinized cells were not agglutinated more strongly by lobster agglutinins; however, increased titers for trypsin-treated red cells were found in the hemolymph of the sea urchin (Ryoyama, 1974) and the sponge (Gold, Phelps, Khalap and Balding, 1974).

The activity of many lectins can be significantly altered by reacting them with various simple sugars prior to exposure to test erythrocytes. N-acetylneuraminic (sialic) acid was the only sugar (of nine tested) which markedly inhibited the tunicate hemagglutinin studied here. Sialic acid-binding lectins have been described in the horseshoe crab (Bird, Uhlenbruck and Pardoe, 1971) and the lobster (Hall and Rowlands, 1974b). *Limulus* lectin (limulin) has been recently reinvestigated by Roche and Monsigny (1974). Limulin was purified and shown to be a glycoprotein of 13.9 S, 335,000 MW consisting of 19,000 MW subunits held together by noncovalent bonds. Agglutination of horse red cells was inhibited by N-acetylglucosamine, free sialic acid, and particularly by glycoprotein-bound sialic acid (human orosomucoid). On this basis it was suggested that the limulin receptor is a complex carbohydrate containing sialic acid and N-acetylglucosamine and is not a monosaccharide.

Johnson (1964) found that N-acetyl-D-galactosamine (GalNAc) and N-acetyl-D-glucosamine (GlcNAc) inhibited the agglutinin of the butter clam. McDade and Tripp (1967) reported that no single saccharide would inhibit the agglutination by oyster lectin of all red cell types tested; however, D-galactosamine (GalN), D-glucosamine (GlcN), GlcNAc, and GalNAc inhibited the agglutination of human red cells, while D-ribose inhibited rabbit erythrocytes. Ryoyama (1974) found that 9 sugars (of 36 tested) could inhibit the lectin of the sea urchin *Pseudocentrotus depressus.* The hemagglutinin of the sponge can be inhibited by galactose (Gold, Phelps, Khalap and Balding, 1974). Hall and Rowlands (1974b) found that lobster agglutinins are not only sensitive to sialic acid (NANAc) but are inhibited by N-glycolylneuraminic acid, N-acetylmannosamine, GlcN and GlcNAc.

The physiological significance of this lectin and many other invertebrate hemagglutinins is as yet undefined. It may function as a humoral recognition factor, a property of certain invertebrate lectins originally described by Tripp (1966). However, Fuke and Sugai (1972) found that ascidian (*Styela plicata*) hemolymph did not stimulate *in vitro* phagocytosis of fixed rabbit erythrocytes. Hall and Rowlands (1974a) point out the structural and physical chemical similarities between some invertebrate hemagglutinins and certain nonimmunoglobulin vertebrate agglutinins and suggest that similar molecules may have been preserved during many stages of animal evolution. The contribution of these molecules to the natural defense mechanisms of higher animals has not been determined.

Other possible roles of invertebrate lectins in nature include Ca^{++} transport, particularly in shell-bearing mollusks. They could also function in sugar transport or storage, or could serve for the attachment of glycoprotein enzymes in organized multienzyme systems (Sharon and Lis, 1972). At the present time there is little evidence to support or refute these hypotheses.

We wish to acknowledge the excellent technical assistance of Lois A. Jordan.

The investigation was supported by a grant from the Whitehall Foundation and Grants CRBS-296 from the National Foundation–March of Dimes, CA-08748-09 from the National Cancer Institute, and IA-11843-02 from the National Institutes of Health.

SUMMARY

An active, naturally-occurring panhemagglutinin has been described in the hemolymph of the protochordate *Halocynthia pyriformis*. This protein lectin is inactivated by temperatures exceeding 50° C, is active in the range pH 6–10, and is resistant to repeated freezing and thawing. Frozen hemolymph retains full hemagglutinating activity for several months. Activity is not reduced by dialysis against saline; however, serum proteins with total hemagglutinating activity can be precipitated by dialysis against deionized water or by treatment with appropriate ammonium sulfate concentrations. This lectin will not agglutinate human erythrocytes in the absence of Ca^{++}; Ca^{++} potentiates the agglutination of red cells from all other species tested. However, Mg^{++} does not stimulate hemagglutination and will inhibit it at certain concentrations.

Halocynthia pyriformis hemolymph will cause the agglutination of red cells from many avian and mammalian species. Human, rabbit, sheep, swine, goat, calf, ox, and pigeon erythrocytes agglutinate weakly with titers of 2–32; whereas, titers of 64–512 were consistently recorded for the red cells of guinea pig, horse, duck, goose, chicken, and turkey. The lectin was not specific for the ABO blood group antigens of man. The hemagglutinin shows considerable cross-reactivity; adsorption with nonhuman mammalian erythrocytes not only decreases activity toward the adsorbing cells but also alters the agglutination of cells from other species. Treatment of red cells with pronase or trypsin causes markedly increased hemagglutination titers; such treatment allows human erythrocytes to agglutinate in the absence of Ca^{++} in the medium. Incubation of hemolymph with N-acetylneuraminic acid strongly inhibits subsequent hemagglutinating activity, suggesting that sialic acid residues are present in the binding site of the lectin.

LITERATURE CITED

Acton, R. T., J. C. Bennett, E. E. Evans, and R. E. Schrohenloher, 1969. Physical and chemical characterization of an oyster hemagglutinin. *J. Biol. Chem.*, **244**: 4128–4135.

Anderson, R. S., N. K. B. Day, and R. A. Good, 1972. Specific hemagglutinin and a modulator of complement in cockroach hemolymph. *Infect. Immun.*, **5**: 55–59.

Aub, J. C., B. H. Sanford, and M. N. Cote, 1965. Studies on reactivity of tumor and normal cells to a wheat germ agglutinin. *Proc. Nat. Acad. Sci. U. S. A.*, **54**: 396–399.

Bird, G. W., G. Uhlenbruck, and G. I. Pardoe, 1971. Serochemical studies of the specificity of some plant and animal agglutinins. *Bibl. Haematol.*, **38**: 58–67.

Bizot, M., 1971. Hemagglutinin from the snail *Eobania vermiculata*. *Vox Sang.*, **21**: 465–468.

Boyd, W. C., 1963. The lectins: their present status. *Vox Sang.*, **8**: 1–32.

Boyd, W. C., 1970. Lectins. *Ann. N. Y. Acad. Sci.*, **169**: 168–190.

Boyden, S. V., 1966. Natural antibodies and the immune response. *Adv. Immunol.*, **5**: 1–28.

Brown, R., L. R. Almodovar, H. M. Bhatia, and W. C. Boyd, 1968. Blood group specific agglutinins in invertebrates. *J. Immunol.*, **100**: 214–216.

Burger, M. M., 1969. A difference in the architecture of the surface membrane of normal and virally transformed cells. *Proc. Nat. Acad. Sci. U. S. A.*, **62**: 994-1001.

Cooper, E. L., C. A. E. Lemmi, and T. C. Moore, 1974. Agglutinins and cellular immunity in earthworms. *Ann. N. Y. Acad. Sci.*, **234**: 34–50.

Cornick, J. W., and J. E. Stewart, 1973. Partial characterization of a natural agglutinin in the hemolymph of the lobster, *Homarus americanus*. *J. Invertebr. Pathol.*, **21**: 255–262.

Cushing, J. E., 1967. Invertebrates, immunology and evolution. *Fed. Proc.*, **26**: 1666–1670.

Finstad, C. L., G. W. Litman, J. Finstad, and R. A. Good, 1972. The evolution of the immune response. XIII. The characterization of purified erythrocyte agglutinins from two invertebrate species. *J. Immunol.*, **108**: 1704–1711.

Fuke, M. T., and T. Sugai, 1972. Studies on the naturally occurring hemagglutinin in the coelomic fluid of an acidian. *Biol. Bull.*, **143**: 140–149.

Gold, E. R., C. F. Phelps, S. Khalap, and P. Balding, 1974. Observations on *Axinella* sp. hemagglutinin. *Ann. N. Y. Acad. Sci.*, **234**: 122–128.

Hall, J. L., and D. T. Rowlands, Jr., 1974a. Heterogeneity of lobster agglutinins. I. Purification and physiochemical characterization. *Biochemistry*, **13**: 821–827.

Hall, J. L., and D. T. Rowlands, Jr., 1974b. Heterogeneity of lobster agglutinins. II. Specificity of agglutinin-erythrocyte binding. *Biochemistry*, **13**: 828–832.

Hammarström, S., and E. A. Kabat, 1971. Studies on specificity and binding properties of the blood group A reactive hemagglutinin from *Helix pomatia*. *Biochemistry*, **10**: 1684-1692.

Inbar, M., and L. Sachs, 1969. Structural difference in sites on the surface membrane of normal and transformed cells. *Nature*, **223**: 710–712.

Jenkin, C. R., and D. Rowley, 1970. Immunity in invertebrates. The purification of a haemagglutinin to rat and rabbit erythrocytes from the haemolymph of the murray mussel (*Velesunio ambiguus*). *Aust. J. Exp. Biol. Med. Sci.*, **48**: 129–137.

Johnson, H. M., 1964. Human blood group A, specific agglutinin of the butter clam *Saxidomus giganteus*. *Science*, **146**: 548–549.

Marchalonis, J. J., and G. M. Edelman, 1968. Isolation and characterization of a hemagglutinin from *Limulus polyphemus*. *J. Mol. Biol.*, **32**: 453–465.

Matsubara, S., and W. C. Boyd, 1974. Hydrolysis of the lectin of *Otala lactea*. *Science*, **183**: 339.

McDade, J. E., and M. R. Tripp, 1967. Mechanism of agglutination of red blood cells by oyster hemolymph. *J. Invertebr. Pathol.*, **9**: 523–530.

Miller, V. H., R. S. Ballback, G. B. Pauley, and S. M. Krassner, 1972. A preliminary physicochemical characterization of an agglutinin found in the hemolymph of the crayfish *Procambarus clarkii*. *J. Invertebr. Pathol.*, **19**: 83–93.

Naspitz, C. K., and M. Richter, 1968. The action of phytohemagglutinin *in vivo* and *in vitro*, a review. *Prog. Allergy*, **12**: 1–85.

NOGUCHI, H., 1903. On the multiplicity of serum haemagglutinins of cold blooded animals. *Zentralbl. Bakteriol. Parasitenkd. Abt. I Orig.* **34**: 286.

PAULEY, G. B., 1973. An attempt to immunize the blue crab, *Callinectes sapidus,* with vertebrate red blood cells. *Experientia,* **29**: 210–211.

PAULEY, G. B., 1974. Physiocochemical properties of the natural agglutinins of some mollusks and crustaceans. *Ann. N. Y. Acad. Sci.,* **234**: 145–158.

PAULEY, G. B., G. A. GRANGER, AND S. M. KRASSNER, 1971. Characterization of a natural agglutinin present in the hemolymph of the California sea hare, *Aplysia californica. J. Invertebr. Pathol.,* **18**: 207–218.

PEMBERTON, R. T., 1970a. Blood group A reactive substance in the common limpet *(Patella vulgata). Vox Sang.,* **18**: 71–73.

PEMBERTON, R. T., 1970b. Haemagglutinins from the slug *Limax flavus. Vox Sang.,* **18**: 74–76.

PROKOP, O., G. UHLENBRUCK, A. ROTHE, AND E. COHEN, 1974. Protectins: Past, present problems, and perspectives. *Ann. N. Y. Acad. Sci.,* **234**: 228–231.

ROBBINS, J. H., 1964. Tissue culture studies of the human lymphocyte. *Science,* **146**: 1648–1654.

ROCHE, A-C., AND M. MONSIGNY, 1974. Purification and properties of limulin: A lectin (agglutinin) from hemolymph of *Limulus polyphemus. Acta. Biochem. Biophys.,* **371**: 242–254.

RYOYAMA, K., 1974. Studies on the biological properties of coelomic fluid of sea urchin. II. Naturally occurring hemagglutinin in sea urchin. *Biol. Bull.* **146**: 404–414.

SCOTT, M. T., 1971. A naturally occurring hemagglutinin in the hemolymph of the American cockroach. *Arch. Zool. Exp. Gen.,* **112**: 73–90.

SELA, B. A., H. LIS, N. SHARON, AND L. SACHS, 1970. Different locations of carbohydrate-containing sites in the surface membrane of normal and transformed mammalian cells. *J. Membr. Biol.,* **3**: 267–279.

SHARON, N., AND H. LIS, 1972. Lectins: cell-agglutinating and sugar-specific proteins. *Science,* **177**: 949–959.

TRIPP, M. R., 1966. Hemagglutinin in the blood of the oyster, *Crassostrea virginica. J. Invertbr. Pathol.* **8**: 478–484.

TYLER, A., 1946. Natural heteroagglutinins in the body fluids and seminal fluids of various invertebrates. *Biol. Bull.,* **90**: 213–219.

UHLENBRUCK, G., U. REIFENBERG, AND O. PROKOP, 1971. Resistance to proteases of *Helix pomatia* anti-A: consequences for tumor cell A-like antigen. *Acta Biol. Med. Ger.,* **27**: 455–457.

WRIGHT, R. K., 1973. Immunobiological studies of the ascidian urochordate *Ciona intestinalis* Linn. *Ph.D. Dissertation, University of California at Santa Barbara,* 164 pp.

WRIGHT, R. K., 1974. Protochordate immunity. I. Primary response of the tunicate *Ciona intestinalis* to vertebrate erythrocytes. *J. Invertebr. Pathol.,* **24**: 29–36.

Reference: *Biol. Bull.*, **148**: 370–379. (June, 1975)

NON-EQUIVALENCE FOR BEAN SEEDS OF CLOCKWISE AND COUNTERCLOCKWISE MAGNETIC MOTION: A NOVEL TERRESTRIAL ADAPTATION?[1]

FRANK A. BROWN, JR. AND CAROL S. CHOW

Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201, and Marine Biological Laboratory, Woods Hole, Massachusetts

Jones (1960) reported that plant growth could be altered by uniform daily rotation. Clockwise rotation depressed growth and counterclockwise accelerated it. The basis of this phenomenon has remained unexplained.

Brown and Chow (1973a, b), investigating water uptake by bean seeds, reported that rate of water uptake varied from day to day in a manner unaccountable in terms of variations in any obvious environmental factors and yet two populations of samples concurrently investigated at two different sites could exhibit between them a high positive correlation ($r = 0.8$) under some circumstances and under other conditions a negative one. It was postulated that beans in their rate of water uptake could correlate either plus (+) or minus (−) with some biologically effective, subtle, uncontrolled geophysical parameter and that other ambient electromagnetic environmental conditions could influence the sign of the correlation.

It was reported further (Brown and Chow, 1973a) that beans in water in closely juxtaposed vessels could mutually bias one another to adopt opposite signs of their correlating relationship under some circumstances and the same sign under others. Beans in paired vessels on rotating platforms (6 rpm) were influenced in these interactions as follows: clockwise rotation favored mutual biasing to the same sign and counterclockwise rotation, to opposite sign. Interaction between beans was reported to be abolished in the weak field of a slowly rotating (1 rpm, clockwise from above) horizontal bar magnet.

An investigation of 'spontaneous' activity in hamsters maintained on platforms undergoing daily rotation (Brown and Chow, 1974) disclosed that these mammals, like the several kinds of plants studied by Jones and by Brown and Chow, are also influenced in opposite manner by clockwise and counterclockwise rotations and display a negative correlation between them in their systematic fluctuations in mean daily activity. Therefore, mammals, like the beans, were presumed to be capable of assuming either of two states, plus (+) or minus (−), in their day to day correlation with uncontrolled pervasive geophysical parameters and that clockwise rotation, other factors equal, favors the opposite sign from counterclockwise.

EXPERIMENTS AND RESULTS

The following exploratory experiment was initially designed to learn whether a rotating magnetic field would influence the rate of water uptake in beans and

---

[1] This research was supported by grants from the National Science Foundation, #GB-31040 and #GB-41392X.

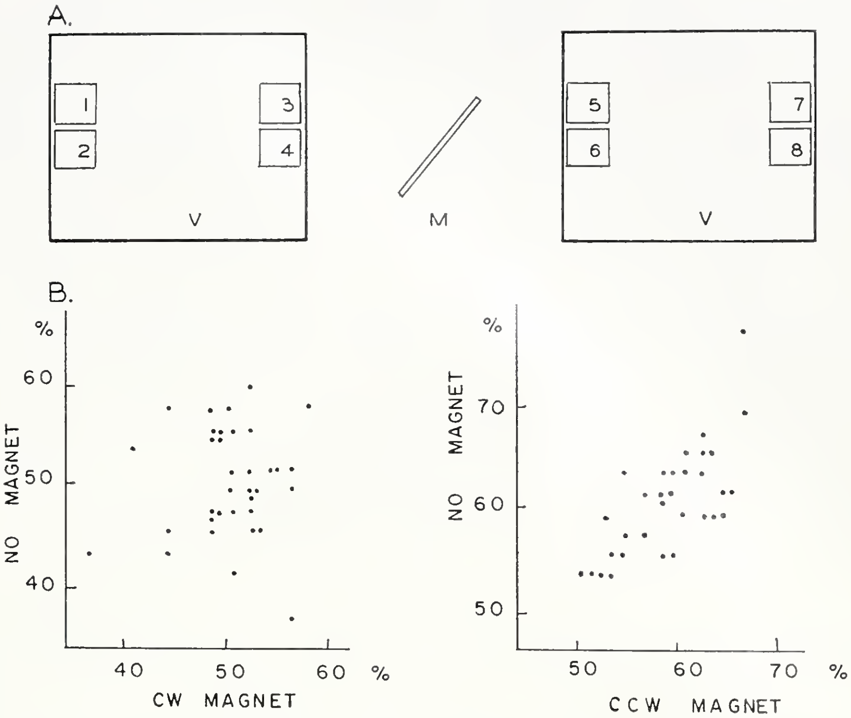


Figure 1. A) The experimental arrangement used in the initial study. 1–8 are eight 20-bean samples in aluminum screen trays; V, large, plastic vessels; M, rotating, 18-cm, Alnico bar magnet (present for experimentals but absent for controls. B) Scatterplot for relationship between day-to-day values for controls, and experimentals in field of a clockwise-rotating magnet, and the same for a counterclockwise-rotating magnet. The values on the axes indicate the percentage increase in weight of the seeds for 4 hours in water.

if so, in what manner and degree. The surprising outcome, however, appears to have revealed a reference environmental parameter which has been involved in the differentiation by organisms of clockwise and counterclockwise rotational directions. In each of two areas in a large laboratory room, about 15 meters apart, four pairs of 20-bean groups in shallow 6 × 6 cm aluminum screen baskets (Figure 1A, 1–8) were arranged in water in two large plastic vessels (Figure 1A, V). For one group an 18 cm rotating (2 rpm) Alnico bar magnet was centered (Figure 1A, M) between the two large vessels. The other 4-pair setup was a duplicate except for absence of a magnet, and comprised the control. All beans came from the same initial stock supply. The temperature of the air-conditioned laboratory was regulated to less than ±1° C.

On each of 35 days distributed over the period May 15 through July 3, 1972, the percentage weight increase through water uptake in beans was determined during a precisely timed initial 4 hours following submergence. This was accomplished through measurement of initial and final wet weights. The amount of water absorbed was expressed as a percentage increase in weight of the initial dry seeds. The 4-hour period always spanned the noon hour. For the experimentals (with magnet) the direction of rotation was clockwise (viewed from above). During an additional 32 days over the interval July 4 through August 18, 1972, the experiment differed only in that the direction of magnet rotation was counterclockwise. The strength of the horizontal vector of the experimental field effected, at the experimental location, about a ±80° oscillation in a compass needle at the positions of the outermost pairs and effected, of course, a 360° complete slave rotation at the location of the innermost pairs.

Correlating the day by day mean rates of water uptake for the experimentals with that of the concurrent controls for the interval when magnet rotation was clockwise (Figure 1B) gave a standard deviation for the controls ±5.18%, experimentals ±4.40%, and $r = +0.070 \pm 0.169$. In short, there was no significant correlation between the two groups. When, however, the possibility of a correlation was similarly examined for the interval when the magnet rotation was counterclockwise (Figure 1B), the standard deviation for the controls was ±5.19%, for experimentals ±4.58%, and $r = +0.729 \pm 0.083$, a very highly significant correlation. Transforming the r's to z's to facilitate determination of statistical significance of the difference yielded, respectively, $+0.070 \pm 0.176$ and $+0.927 \pm 0.185$. The difference between these two was 0.857 ± 0.255, a value highly significantly different from zero ($P < 0.001$).

Providing a parallel, secondary control over the total period of the rotating magnet study, located in the same large laboratory room, and involving beans from the same stock supply were two more experimental series, intermingled with one another, each with six aluminum-screen baskets containing 20 beans. For these, each basket was submerged in a separate small plastic vessel. One group comprised three pairs of juxtaposed vessels; the other group constituted six vessels each separated from any other by at least 70 cm. The day-to-day means for all twelve of these vessels of beans over the period of the present investigation were calculated. When these values were correlated with those of the concurrent specific primary controls for the rotating magnet series a strong positive value was obtained. This positive correlation between the primary and secondary controls continued unmodified throughout the intervals when the correlation was abolished by the clockwise magnet rotation and was unaffected by the counterclockwise.

Despite the slightly different experimental set-ups, from May 15 through August 18 the two controls, primary and secondary, correlated strongly over the whole period, $r = +0.738 \pm 0.055$ ($N = 67$). They also correlated highly significantly over *both* the period of clockwise magnet rotation ($r = +0.527 \pm 0.122$, $N = 35$) and counterclockwise rotation ($r = +0.603 \pm 0.113$, $N = 32$), exhibiting no significant difference in the degree of plus correlation between the two periods. Standard deviation for the secondary controls over these two periods were, respectively, ±5.03% and ±5.54%, again with neither a significant difference between one another nor with the primary controls.

All these findings support the assumption that the times of the two rotating-magnet series were essentially equivalent to one another and did not alter qualitatively between the two successive segments of the year. These comparative values also support the presumption that the observed abolition of the correlation was actually effected by the presence of the clockwise-rotating magnetic field, an effect not similarly produced by the counterclockwise field during which time no statistically significant difference occurred between the r of experimental *vs.* primary control ($+0.729 \pm 0.083$) and the r of the primary *vs.* the secondary control ($+0.603 \pm 0.113$).

While it could not be concluded unequivocally that both clockwise and counterclockwise magnet rotation would have abolished the correlation during the first period and neither would have done so during the second period this seemed improbable and to be a postulation unnecessarily complex at this juncture. A second experiment, performed to check directly the foregoing conclusions, removed this doubt.

This second experiment was performed between January 21 and May 2, 1974, with another lot of beans with significantly lower mean rate of water uptake. For this one, three large circular wooden tables were used. These were at least five meters from one another. Two of the tables had at their centers an 18 cm horizontal bar magnet; one rotated clockwise two rpm, and the other counterclockwise one rpm. The third table, without a magnet, served as a simultaneous control (Figure 2A). Four pairs of small plastic dishes each containing 20 beans in water were placed equidistantly around and 40 cm from the center of each table. The experimental horizontal magnetic fields at the positions of the beans were close in strength to the earth's. A total of 72 such experiments were run on as many days over the three-month period.

Correlating all 72 values of mean bean water-uptake rates on each of the two tables bearing the rotating magnets, with the controls, yielded the scatterplots depicted in Figure 2B. The coefficient for the clockwise magnet was $+0.0135$ and for the counterclockwise magnet was $+0.397$. Transforming these values to z, the difference between them was 0.406 ± 0.169 ($P < 0.02$). Excluding for the clockwise table, those twelve values lying below 14.0 and above 23.0 the coefficient became negative, $r = -0.267$, and the difference between the z's was 0.694 ± 0.178 ($P < 0.001$). This manner of exclusion of values would not itself be expected to impose any correlation upon truly random data.

The difference between the variances for beans within the clockwise and counterclockwise rotating fields of the magnets was significant ($F = 1.79$; $P < 0.01$). It was not identifiable in the immediate study whether this difference arose from the direction of rotation of the magnet *per se,* or from other specific characteristics of the particular laboratory location.

Other experiments with bean water-uptake on rotating platforms and in the fields of rotating magnets were performed during the summer of 1974 at the Marine Biological Laboratory, Woods Hole, Massachusetts. The room was air-conditioned to relatively constant temperature, $22 \pm 1\frac{1}{2}°$. Special care was taken in timing the four-hour runs in water; the beans were all counted out in their screened trays, and labeled, in advance of their submersion in water. The submersions were made in sequence around the tables, while all systems were operating, one after another at one minute (± ten seconds) intervals. Exactly four hours later ± ten seconds, each tray was assayed, similarly one minute apart. Five tables, about 92 cm in diameter, were employed on a wooden bench. Four possessed a reversible gearmotor, electrically driven, to rotate either the whole table or an 18 cm bar magnet at its center. The directions of rotation, clockwise and counterclockwise, were

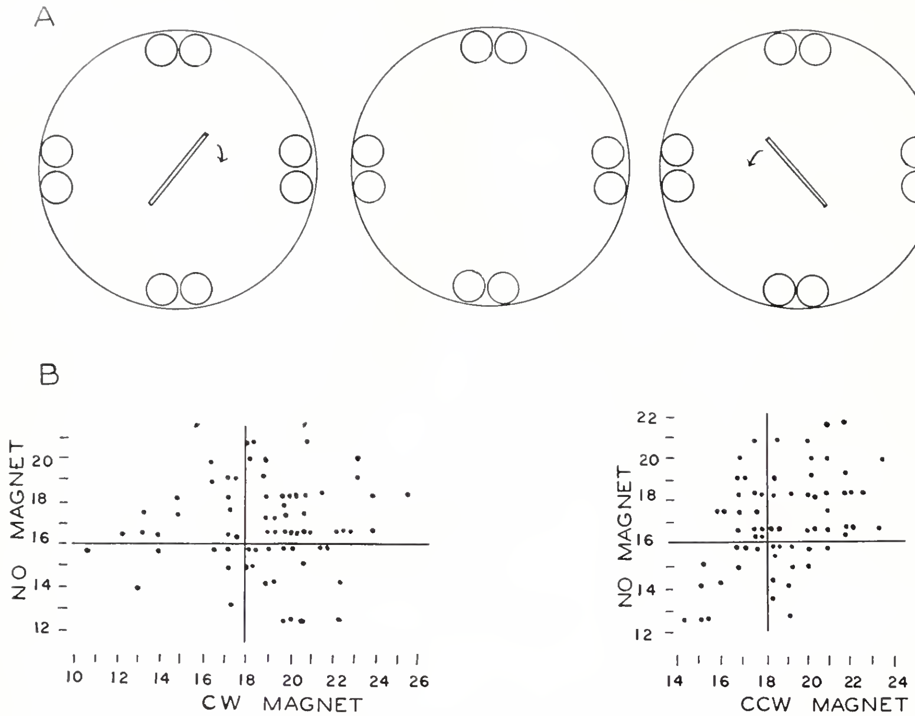


FIGURE 2. A) The three conditions concurrently operating in the second experiment. Small circles on the larger tables indicate plastic dishes (9 cm in diameter) each containing 20 beans in aluminum screen trays. B) Scatterplots show relationships between day-to-day percentage values for clockwise and counterclockwise tables relative to the common controls.

alternated between the two rotating tables and two rotating magnets on consecutive days to compensate for any position effect.

On fifty days, five days a week between June 17 and August 23, 1974, experiments including both rotating tables and rotating magnets (both, 1 rpm) were performed. There were four pairs of vessels distributed around the edge on each table. There was also a comparable control table, non-rotating and without a magnet. The day-by-day data were converted to deviations from the daily means of all sixty vessels of beans being measured concurrently in four experiments in the room because of relatively substantial systematic variations occurring in water uptake rates over the summer.

When the means for the control and clockwise rotating tables were correlated (with one of the 50 points which was widely discontinuous with the mass of points, omitted), the clockwise table gave a very low negative value, $r = -0.078$ (Figure 3A), while the counterclockwise table yielded a statistically highly significant negative value, $r = -0.534$ (Figure 3B). After transforming these to z's, the difference between them was found to be 0.518 ± 0.208, $P < 0.02$.

Now, doing the same for the rotating magnet series, counterclockwise magnet rotations (Figure 3C) gave a positive but statistically insignificant value, $r =$

+0.144. Clockwise magnet rotation, on the other hand, yielded a still smaller positive value, $r = +0.039$ (Figure 3D). Indeed, the variance on the clockwise magnet table was higher than for the counterclockwise one ($F = 1.83$; $P < 0.05$), just as had been discovered for a comparable experiment performed earlier in Evanston. However, an inspection of the scatterplot of the relationship suggested that as the clockwise values increased above, or fell below, the mean of the values obtained for the day the values of the stationary controls increased proportionately. Indeed, correlating the values of the clockwise magnet table, without regard to sign, with the values from the control table yielded $r = 0.411$, or $z = 0.437 \pm 0.145$.

It is evident that either continuation of the negative correlation of the clockwise beans below their mean, or of the positive correlation above it, would have effected a very substantially altered rate of water uptake relative to the control.

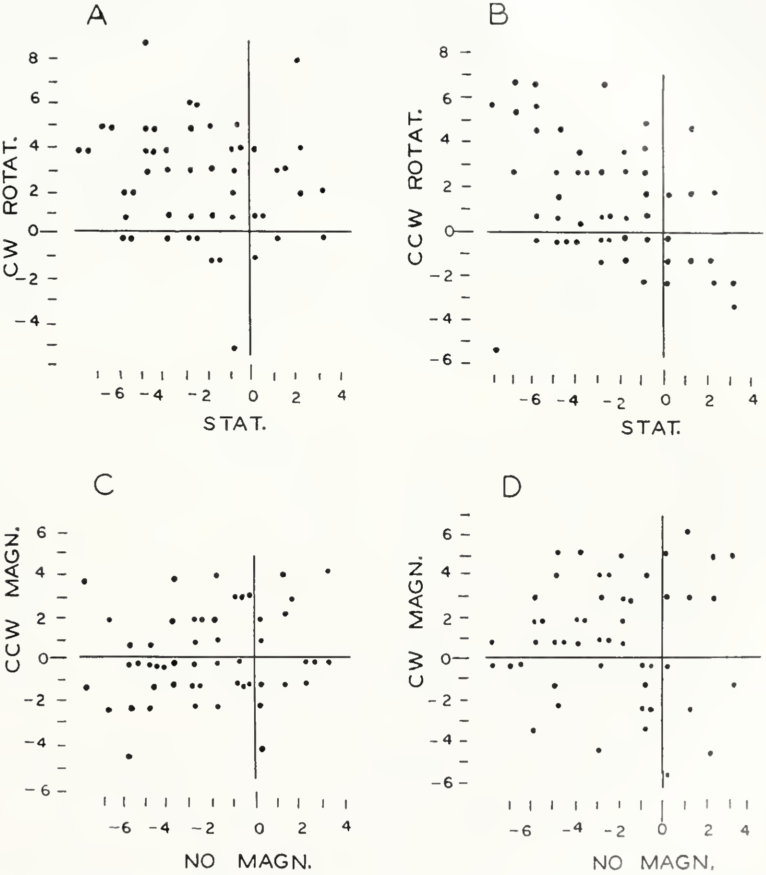


FIGURE 3. Results of studies of beans on rotating (1 rpm) and stationary platforms, and in the field of a very weak rotating magnet (1 rpm) during the summer of 1974. Scatterplot A shows means for those beans on clockwise table *vs.* ones on a stationary one; *B*, same for beans on counterclockwise platform; C, same for beans in field of counterclockwise-rotating magnet; D, same for beans in field of a clockwise-rotating magnet.

The change in sign of the correlation with the exogenous effective parameters seems, therefore, probably to be an adaptive behavior for the maintenance of a relatively constant state.

Most important of all, in this last experimental series, there is a remarkable confirmation and extension of the results obtained earlier in our Evanston laboratory. Counterclockwise magnetic field rotation results in an opposite sign of correlation with the concurrent stationary control to that found for counterclockwise table rotations in the earth's magnetic field (Figure 3, B and C). These two are highly significantly different from one another. The difference between their z's is 0.740 ± 0.206, $P < 0.001$. For the actual values for the two, we must recall that in dealing with deviations from the daily means with groups of the size of the present series instead of with actual values such as could be used for Figures 1 and 2, the null expectation is a negative correlation of the order of about $r = -0.2$. We get, therefore, in this series a direct answer to a hypothesis that for the beans a magnetic field rotating counterclockwise will produce effects which are the opposite of those observed for organisms rotating counterclockwise in the earth's relatively static vector field.

Now examining the clockwise magnetic field effect (Figure 3D), we see an apparent active sign reversing system which results in essentially no overall correlation with the controls. In retrospect, this is what one can note for the same relationships in Figures 1 and 2. Clockwise rotation of a magnetic field appears to unstabilize the sign of response, possibly in part by dissociating the organism from environmental factors which normally determine it. The distributions of points in scatterplots 3A and D are quite different one from the other, though only dubiously suggesting opposite effects.

Discussion

The asymmetrical magnetic response is consistent with the previously described differences between influences of clockwise and counterclockwise platform rotation in the earth's natural atmosphere for which, incidentally, no hypothesis as to causative basis has been advanced up to the present. It is also consistent with the earlier demonstrations in a number of laboratories of organismic responsiveness to vector directional differences in extremely weak magnetic fields in which organisms were used ranging from Paramecium (Brown, 1962) and Volvox (Palmer, 1963) through worms and snails (Brown, 1971) and insects (Picton, 1966; Lindauer and Martin, 1968; Wehner and Labhart, 1970) to European robins and homing pigeons (Wiltschko and Wiltschko, 1972; Walcott and Green, 1974), fishes (Becker, 1974), and gerbils (Stutz, 1971).

In the present studies, clockwise rotation of the magnet appeared to render essentially random, or forced to opposite sign in part or in whole, the relationship of bean water uptake to the effective concurrent geophysical fluctuations, resulting in nearly equal distribution of plus (+) and minus (−), or a preponderance of opposite, signs in correlating state in the clockwise magnet experimentals relative to the controls. On the other hand, counterclockwise rotation of the magnet had little or no influence on the correlating sign which was therefore predominantly free to be determined by environmental parameters which parallelly influenced the experimentals and controls.

These results are consistent with an earlier published report (Brown and Chow, 1973a) that members of rotating pairs tend to adopt the same sign when on a platform undergoing clockwise rotation if we postulate that counterclockwise magnet rotation provides an influence the equivalent of clockwise platform rotation in the earth's magnetic field. And contrariwise, clockwise magnet rotation provides the equivalent of counterclockwise platform rotation which was earlier reported to result in a statistically significant reduction of plus correlation between members of pairs. The results also confirm earlier findings that clockwise magnet rotation abolished correlations between members of paired dishes of beans.

A hypothesis is supported that counterclockwise magnet rotation at 1 rpm has relatively little effect on the sign determination of the organism's correlation with fluctuations in effective but still unidentified atmospheric field parameters. A positive correlation is evident with untreated controls. Quite the opposite effect is produced by counterclockwise rotation of the organisms at the same place and time; this now effects the opposite sign, or a negative correlation with non-rotating controls.

Whereas simultaneous clockwise and counterclockwise table rotations at 1 rpm tend to yield opposite results relative to one another, that is, clockwise table rotation favors a correlation with controls which is basically positive, the clockwise magnetic field rotation provides a correlation which in three experiments was of lower positive value, and indeed, one in which the structure of scatterplots seems to contain a good degree of a negative relationship within them. Therefore, all the results of these experiments are consistent with a hypothesis that effects noted on clockwise and counterclockwise rotating tables are in good measure a consequence of the organism steadily and systematically being altered in their orientation to the geomagnetic fields. It is predicted that further experiments will give firm support to this hypothesis.

All these findings also provide support for the postulation that altered, extremely weak, electromagnetic fields have been responsible for the previously described either low positive or significant negative correlations between bean water uptake inside and just outside a metal-sheathed walk-in constant temperature chamber (Brown and Chow, 1973a, b), under otherwise apparently similiar conditions. They also provide support for a hypothesis that the rather substantial reported differences in hamster activity (Brown and Chow, 1974) between two rotational directions, at one revolution per day, are effected by the continuous clockwise and counterclockwise animal motions relative to the geomagnetic field.

Such differences in effects between directions of magnetic motion, often even effecting essentially opposite responses, constitute still another reason why the capacity of living creatures to sense the terrestrial electromagnetic fields so long eluded disclosure. At the same time, such a particularized difference encourages speculation that such weak atmospheric electromagnetic forces are playing vital roles for living systems in biological regulations including the enigmatical clocks. We postulate that the asymmetrical magnetic response is an adaptation of life to its rotating and sun-orbiting planetary environment.

Quite a substantial range of variation in rates of a biological process continuing in what have earlier been presumed to be constant conditions for organisms appear able to result from responsiveness to subtle, pervasive geophysical parameters. It is evident, therefore, that such a capacity as that for a sign-changing relationship to fluctuations in atmospheric electromagnetic fields can be a very effective contributing factor for homeostasis. It is interesting to note that the differing mean rates of water uptake for both experimentals and controls evident in Figure 1B depict two portions of a persistent annual variation which has now been followed through two years in two different lots of beans, and which simultaneously contains a significant mean synodic monthly component, as well.

The authors wish to acknowledge the excellent assistance of King-Tim Mak and John Wandel in obtaining data for the Evanston series, and of Michele Lorand and Tom Moore for the Woods Hole series.

SUMMARY

1. Three experiments on water-uptake in pinto beans, *Phaseolus vulgaris,* during their first four hours in water have all indicated that clockwise and counterclockwise rotating magnetic fields have statistically significantly different effects upon the rate of the process.

2. The results suggest that this difference is independent of time and place. Critically performed experiments at Evanston, Illinois and Woods Hole, Massachusetts, over more than a two-year span, all gave essentially the same results.

3. A clockwise rotating magnetic field abolished, for the beans, a correlation with concurrent control beans without magnet, while a counterclockwise rotating magnet failed to do so.

4. In a concurrent series involving rotating beans (1 rpm, clockwise and counterclockwise) and beans in fields of a weak rotating magnet (1 rpm, clockwise and counterclockwise), the effects of counterclockwise magnet and counterclockwise table rotations were of opposite character and highly significantly different from one another. Effects of clockwise magnet and clockwise table rotations differed significantly, in turn, from their opposite directions of rotations.

5. The results uniformly support the hypothesis that the different effects reported between clockwise and counterclockwise rotation of organisms result from their systematic, directional motions relative to the geomagnetic field.

6. It is postulated that this non-equivalence for organisms of clockwise and counterclockwise rotations of such extremely weak magnetic fields reflects a novel and fundamental adaptation of organisms to their rotating and sun-orbiting environments.

LITERATURE CITED

BECKER, G., 1974. Einfluss des Magnetfelds auf das Richtungsverhalten von Goldfischen. *Naturwissenschaften,* **61**: 220–221.

BROWN, F. A., JR., 1962. Responses of the planarian, *Dugesia,* and the protozoan, *Paramecium,* to very weak horizontal magnetic fields. *Biol. Bull.,* **123**: 264–281.

BROWN, F. A., JR., 1971. Some orientational influences of nonvisual, terrestrial electromagnetic fields. *Ann. N. Y. Acad. Sci.,* **188**: 224–241.

BROWN, F. A., JR., AND C. S. CHOW, 1973a. Interorganismic and environmental influences through extremely weak electromagnetic fields. *Biol. Bull.,* **144**: 437–461.

BROWN, F. A., JR., AND C. S. CHOW, 1973b. Lunar-correlated variations in water uptake by bean seeds. *Biol. Bull.,* **145**: 265–278.

Brown, F. A., Jr., and C. S. Chow, 1974. Phase shifting an exogenous variation in hamster activity by uniform daily rotation. *Proc. Soc. Exp. Biol. Med.*, **145**: 7–11.

Jones, R. L., 1960. Response of plants to a uniform daily rotation. *Nature*, **185**: 775.

Lindauer, M., and H. Martin, 1968. Die Schwereorientierung der Bienen unter dem Einfluss des Erdmagnetfeldes. *Z. vergl. Physiol.*, **60**: 219–243.

Palmer, J. D., 1963. Organismic spatial orientation in very weak magnetic fields. *Nature*, **198**: 1061–1062.

Picton, H. D., 1966. Some responses of *Drosophila* to weak magnetic and electrostatic fields. *Nature*, **211**: 303.

Stutz, A. M., 1971. Effects of weak magnetic fields on gerbil spontaneous activity. *Ann. N. Y. Acad. Sci.*, **188**: 312–323.

Walcott, C., and R. P. Green, 1974. Orientation of homing pigeons altered by a change in the direction of an applied magnetic field. *Science*, **184**: 180–182.

Wehner, R., and Th. Labhart, 1970. Perception of the geomagnetic field in the fly, *Drosophilia melanogaster*. *Experientia*, **26**: 967.

Wiltschko, W., and R. Wiltschko, 1972. Magnetic compass of European robins. *Science*, **176**: 62.

Reference: *Biol. Bull.*, **148**: 380–392. (June, 1975)

CHEMORECEPTION IN THE SHRIMP, *PALAEMONETES PUGIO*: COMPARATIVE STUDY OF STIMULATORY SUBSTANCES IN HUMAN SERUM[1]

WILLIAM E. S. CARR AND SAMUEL GURIN

C. V. Whitney Marine Laboratory, of the University of Florida at Marineland,
Rt. 1, Box 121, St. Augustine, Florida 32084

There is evidence that several aspects of the behavior of marine crustaceans are influenced or directed by external chemical agents. Studies with various crustaceans have indicated that external chemical agents are involved with such diverse phenomena as feeding behavior (Case and Gwilliam, 1961; Crisp, 1967; Laverack, 1963; and others), host location by commensals (Ache and Case, 1969; Davenport, 1966), mate recognition (Kittredge, Terry and Takahashi, 1971; Ryan, 1966), and prey concealment (Kittredge, Takahashi, Lindsey and Lasker, 1974). Chemical stimulation of feeding behavior has received the attention of many investigators (for review, see Lindstedt, 1971). Effectively all of the studies on this phenomenon in marine crustaceans have focused on the stimulatory capacity of substances of low molecular weight, especially amino acids and amines. Inadequate attention has been given to whether or not the substances studied actually occurred in sufficient concentrations in potent preparations such as extracts or body fluids to account for the responses given by the experimental animal to the latter preparations.

The report that follows has a twofold purpose: 1) to describe a procedure for studying a chemically mediated feeding response in the shrimp, *Palaemonetes pugio,* and 2) to account for the response-inducing capacity of human serum in terms of the compounds present and their relative concentrations in serum. Human serum was selected as the standard for this initial study with *P. pugio* because in addition to being a response-inducer, a large literature exists on its chemical composition and many of its principal components are available commercially. This report also includes some preliminary results concerning the nature of the major stimulants present in certain other body fluids and extracts.

METHODS

Maintenance of animals

Groups of several hundred *Palaemonetes pugio* were collected every two weeks with a dip net near the Whitney Marine Laboratory. Each group was maintained in a 35-gal aquarium provided with running filtered sea water, aeration, and a thin layer of fragmented beach shell on the bottom. An acclimation period of 14 to 18 days in the laboratory was required before each new group could be used in experiments. During the acclimation period the group was fed daily with

---

[1] This research was supported in part by Grant No. GB-39289 from the National Science Foundation.

TetraMin (Tetra Sales Corp., Hayward, Calif.). After the initiation of experiments the group was fed once a week. Another source of food consisted of recently molted individuals which were cannibalized regularly. Following the acclimation period, experiments with a group of shrimp continued over a two-week period after which the group was replaced. Individual shrimp were used in no more than one bioassay per day.

Bioassay procedures

Bioassays were conducted in 27 × 17.5 × 4 cm plastic boxes (Plano Molding Co., Plano, Ill.) divided by partitions into six compartments of equal size. Each box was placed inside a wooden frame over which a plexiglass sheet was mounted. Solutions to be tested were introduced into each compartment through a Bubble Cone aerator (Marineland Aquarium Products, Aquaria, Inc., Los Angeles). The Bubble Cone (referred to hereafter as the "target") was affixed to a section of 3-mm Tygon tubing and entered each compartment through a hole (16-mm dia) in the top of the box. Holes (13-mm dia) in the plexiglass sheet above each box received a No. 00 rubber stopper bored to accommodate the Tygon tubing. This arrangement permitted the target to be introduced into each compartment with minimal disturbance to the shrimp and to be secured in a stationary position with the tip approximately 1 cm from the bottom of the box. Shrimp could move freely beneath the tip of the target. A 5-ml pipette affixed to the Tygon tubing and held in place by a burette clamp served as the reservoir for each solution to be introduced through the target. Flow rate was regulated with a screw clamp and stopped or started with a pinch clamp. The pipette was refilled after each test by suction with a rubber bulb.

Bioassays were standardized as follows. (1) Each plastic box was prerinsed in sea water and to each compartment was added 100 ml of water from the aquarium containing the shrimp to be used. Three shrimp were transferred by dip net into each compartment to give a total of 18 shrimp per box (one test group). (2) Each box of shrimp was placed in its wooden frame and left undisturbed for 60 minutes. When a solution was to be tested at several concentrations, several boxes of shrimp were prepared as above at 25 minute intervals so that each successive box remained undisturbed for 60 minutes and was ready for testing shortly after completing tests with the preceding box. (3) A single concentration of a solution was used for each entire box of shrimp. The solution was introduced into each compartment for a 1.5 minute period at a flow rate calibrated to deliver 3.6 to 4.0 ml during this period. The response of shrimp to stimulatory solutions is described in the results. The pipette, tubing and target were rinsed thoroughly in deionized water and filtered sea water before and after each concentration of a solution was tested. All solutions were prepared in the same filtered sea water that was provided to the aquarium maintaining the shrimp. During bioassays, compartments containing injured or newly molted shrimp were not tested.

Preparation of solutions for bioassay

For testing single compounds or mixtures of compounds, a concentrated stock solution was prepared in filtered sea water and kept in ice. When necessary the pH was adjusted to 7 to 8 as determined with Alkacid paper. A solution of the desired concentration was prepared by pipetting a small volume of stock solution into the appropriate volume of sea water.

Fresh frozen human serum was obtained from the Clinical Chemistry Laboratory, J. Hillis Miller Health Center, University of Florida. Lyophilized human serum, human alpha$_1$-globulins (Fraction IV-1), beta globulins (Fraction III), gamma-globulins (Fraction II), glycoprotein (orosomucoid, Fraction VI), and albumin were obtained from Miles Laboratories. Purified plasma lipoproteins were prepared by Dr. Richard Triplett, Department of Medicine and Biochemistry, University of Florida.

Ultrafiltration and dialysis

Ultrafiltration through Amicon UM-2 membranes retaining molecules greater than *ca.* 1000 molecular weight was carried out in a 50-ml stirred cell with 30 psi of N_2 at 4° C. Certain protein solutions were prepared in 1% NaCl and dialyzed for 24 hr at 4° C with constant stirring in 20-mm Visking tubing. Dialysis was effected by four changes of one liter of 1% NaCl.

Preparation of body fluids and extract from other sources

All preparations were made from live specimens. Hemolymph serum of male blue crabs (*Callinectes sapidus*), extract of pink shrimp (*Penaeus duorarum*) and oyster fluid (*Crassostrea virginica*) were prepared as described by Carr, Hall and Gurin (1974) and by Gurin and Carr (1971). Extract of spiny lobster (*Panulirus argus*) was prepared by cutting up abdominal muscle in cold deionized water and shaking for 30 minutes in an ice bath followed by centrifugation at 4° C, decantation, and storage in ice. Extract of coquina, *Donax variabilis,* was prepared by grinding up entire animals with a mortar and pestle in cold filtered sea water followed by centrifugation and treatment as above.

Statistical treatment of data and presentation of results

Many of the data which follow were analyzed by Potency Probit Analysis employing the computer program of Daum and Givens (1963) at the Northeast Regional Data Center, University of Florida. A brief description of the program is provided by Carr (1967). Comparisons of the relative potencies of solutions were obtained by assaying solutions over a range of concentrations and determining the concentration at which each solution induced a positive response in 50 per cent of the test animals (effective dose for 50 per cent of test animals = ED_{50}). Unless otherwise indicated each determination of an ED_{50} involved bioassays of at least three concentrations with a minimum of 18 shrimp employed at each concentration. Statements of statistical significance are based on 95% limits of confidence.

In the literature, concentrations of many constituents of human blood are usually expressed in terms of their concentrations in blood plasma. In the current study the concentrations of constituents that are assumed for human serum were obtained from reported concentrations in plasma. Serum is considered to be plasma minus fibrinogen and certain other clotting agents. To compare directly the potency of certain constituents of human serum with that of serum itself, the concentrations of the constituents are expressed in terms of serum milliequivalents. One serum milliequivalent is defined here as the amount of the particular constituent that would be present per ml of sea water when serum itself was diluted to a concentration of one μl per ml of sea water. Hence, one thousand milliequivalents equals the amount of the particular constituent that would be present in full strength serum (*i.e.,* 1000 μl/ml).

RESULTS

In an aquarium individuals of *Palaemonetes pugio* remained for the most part on the bottom in a reasonably stationary position. However the introduction of pieces of fish, shrimp or other potential food items quickly stimulated a marked change in behavior. The initial recognition of the presence of food was indicated by increased movement followed by a rapid swarming around and a distinct searching behavior that included swimming up and down throughout the water column. Food itself was not required for the stimulation of such behavior since the addition of small amounts of filtered extracts would induce the same response. Likewise, small pieces of sponge soaked in an extract would be sought out and converged upon by groups of shrimp. Pieces of clean sponge soaked only in sea water were ineffective. These preliminary observations led to the development of the bioassay procedure used in the current study.

In the plastic boxes used for bioassays, the introduction of a strong stimulant is quickly detected by individuals in a compartment and the target is usually found within a few seconds. Preliminary tests with several hundred individuals over various periods of time revealed that a standardized test period of 1.5 minutes was adequate for obtaining responses from effectively all individuals that would respond at all. A positive response in the bioassay procedure consists of a shrimp moving to the target and grasping it. Since the lower tip of the target was *ca.* one cm from the bottom of the compartment, each positive response required that the shrimp leave the bottom and suspend himself near the surface when the target is grasped. This behavior was very seldom observed in the absence of stimulant. In controls that were run during the course of these experiments, only two individuals out of 440 (0.5%) gave positive responses when filtered sea water alone was introduced through the target.

Tests with glycine

During the initial stages of this investigation, it was noted that glycine was a response inducer. Hence glycine was used to determine the degree of reliability of the procedure. Fresh glycine solutions were tested repetitively on four separate days with individuals from a single group of shrimp and with individuals from four separate groups collected at different times during a period of three months. With individuals from the same group of shrimp, the ED_{50} of glycine averaged 3.7×10^{-3} M and ranged from 2.4×10^{-3} to 5.2×10^{-3} M. These determinations were not significantly different. With individuals from separate groups the ED_{50} of glycine averaged 4.9×10^{-3} M and ranged from 2.8×10^{-3} to

7.2×10^{-3} M. These determinations were not significantly different. On the basis of these repeated assays of glycine it was evident that the procedures had a satisfactory degree of reliability and could be used to measure the relative potency of solutions. The decision to use a 14 to 18 day period of acclimation prior to the initiation of experiments with a new group of shrimp (see methods) evolved primarily from the above studies with glycine. Shrimp kept in the lab for shorter periods did not adjust well to confinement in the plastic boxes. Many would continue to scurry about in the compartments and jump against the walls even after a one-hour period. Shrimp in this hyperactive condition would frequently even refuse to accept food and could not be tested. However, after 14 to 18 days in captivity this hyperactive response to transfer and confinement decreased dramatically and by the end of a one-hour period effectively all individuals had assumed a reasonably inactive state.

TABLE I

Concentrations of substances in human plasma and responses of P. pugio *to these substances.*

Substance(s)	Upper limit of normal conc. in human plasma (mg/ml of plasma)	Concentrations tested		Per cent of shrimp responding
		mg/ml	serum milliequivalents per ml	
Albumin	45†	1.0	22	22
		5.0	110	28
Alpha$_1$-globulins	6†	0.12	20	11
		0.6	100	50
		1.5	250	56
Orosomucoid	0.75†	0.075	100	7
		0.375	500	33
Beta-globulins	11†	0.11	10	17
		1.1	100	11
Gamma-globulins*	15†	0.15	10	6
		0.6	40	17
		1.2	80	33
High density lipoprotein	5.3‡	0.33	60	13
		0.66	120	17
Low density lipoprotein	4‡	0.14	35	27
		0.28	70	53
		0.56	140	56
Very low density lipoprotein	2‡	0.04	20	13
		0.4	200	50
Glycogen	0.06†	0.1	1700	6
		1.0	17000	56

† Concentrations derived from White, Handler and Smith (1964, pp. 628 and 633). Values for proteins presented by White *et al.* are in units of g/100 ml.

‡ Concentrations derived from Scanu and Wisdom (1972).

* Gamma-globulins dialyzed exhaustively for 24 hours prior to testing to remove glycine added by Miles Laboratories as a stabilizing agent.

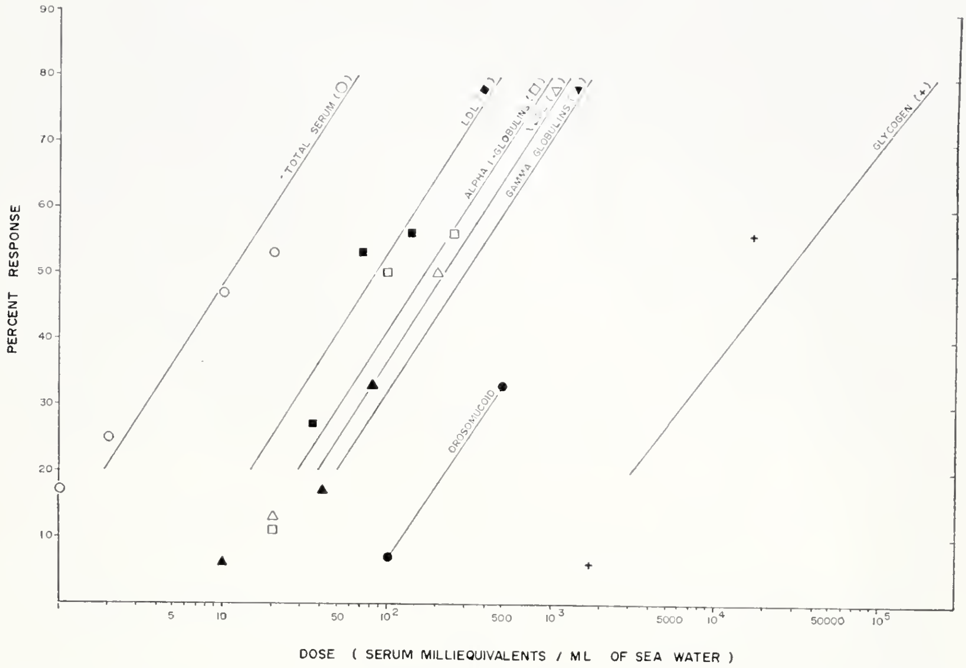


FIGURE 1. Responses given by *P. pugio* to human serum and to certain serum proteins and glycogen. Regression lines were drawn from computed effective dose values obtained by Potency Probit Analysis. The potency (and 95% confidence limits) of low density lipoprotein (LDL) and alpha$_1$-globulins were 0.131 (0.444–0.049) and 0.065 (0.197–0.024) respectively. Potency × 100 equals percent of activity of total serum.

Studies of stimulatory substances in human serum

Following ultrafiltration of human serum through a UM-2 membrane it was found that the retentate containing substances of greater than *ca.* 1000 MW was as active as the total serum. The ultrafiltrate containing smaller molecules was significantly less active and accounted for less than 20 per cent of the activity of the total serum.

In human serum, proteins and glycogen are the major substances with molecular weights greater than 1000. Table I contains the results of bioassays of many of these substances together with the normal concentrations at which they occur in human plasma. All of the substances possessed a certain degree of response-inducing activity. When the concentrations tested are considered solely on the basis of mg per ml of sea water, the activities of five of the substances (alpha$_1$-globulins, orosomucoid, low density lipoprotein, very low density lipoprotein, and glycogen) are quite similar. The ED_{50} values of these five substances were estimated to range between *ca.* 0.3 and 0.8 mg per ml of sea water. Values for the other substances were considerably higher. Since there are, however, differences in the inherent concentrations of these various substances in serum itself, these differences must be considered in assessing the relative contribution that each sub-

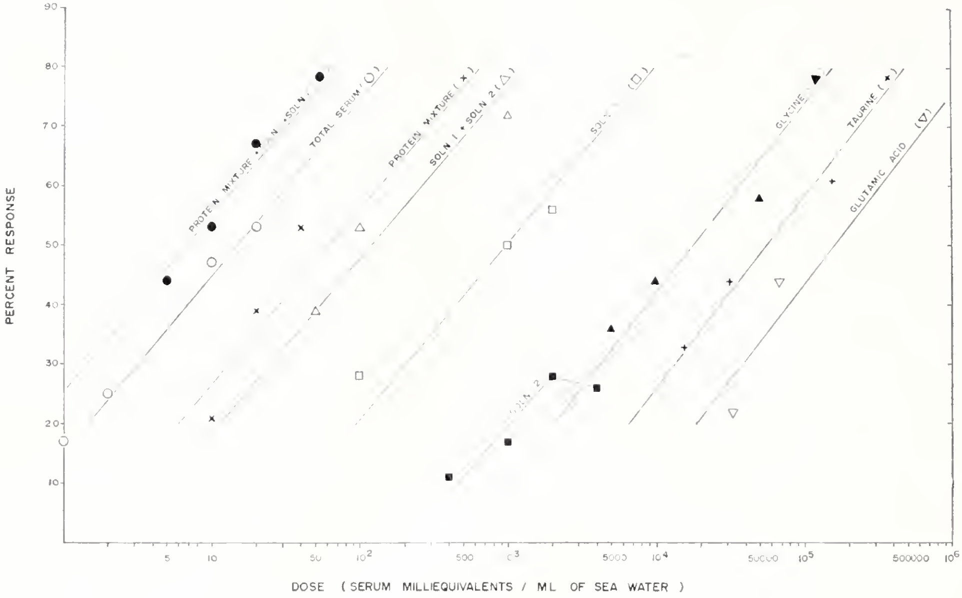


FIGURE 2. Responses given by *P. pugio* to human serum and to certain serum components and mixtures of components. Regression lines were drawn from computed effective dose values obtained by Potency Probit Analysis. No regression line was computed for results obtained from Solution 2. Potencies (and 95% confidence limits) of major mixtures were as follows: Protein mixture + Solution 1 + Solution 2 = 2.226 (15.311–0.659); Protein mixture = 0.239 (0.875–0.066); Solution 1 + Solution 2 = 0.127 (0.874–0.035); Solution 1 = 0.016 (0.073–0.005). Potency × 100 equals percent of activity of total serum.

stance(s) might make to the potency of serum. Differences in inherent concentrations are illustrated in Figure 1 which expresses all concentrations in terms of serum milliequivalents (see methods). The figure illustrates the potency of certain of these serum components relative to the potency of serum itself. Individually, each type of protein (and glycogen) could account for only a small portion of the potency of total serum. Low density lipoprotein and the alpha$_1$-globulins were the most potent of these substances and accounted for approximately 13 per cent and 7 per cent of the potency of serum respectively. The other substances, though all possessing various degrees of activity could, individually, account for less than 5 per cent of the potency of total serum.

The finding that none of the separate groups of proteins (or glycogen) cited above was able to account for a major part of the potency of total serum suggested that the potency of serum might be attributed to a mixture of two or more of these groups. A mixture of human albumin, alpha$_1$-globulins, beta globulins, gamma-globulins, low density lipoprotein and orosomucoid was prepared such that each constituent was present at the same relative concentration as in serum. This mixture was dialyzed exhaustively and bioassayed. Figure 2 shows that this mixture was somewhat more active than any of its individual components and could account for approximately 24 per cent of the potency of the total serum.

Table II gives the composition of stock solutions that were prepared for measuring the potency of mixtures of the 37 major organic constituents of low molecular weight found in human plasma. For convenience these substances were divided into two groups for testing: Solution 1—comprised of amino acids, taurine, and urea; Solution 2—comprised of other small nitrogenous substances, carbohydrates, and organic acids. Figure 2 shows that the mixture of substances in Solution 1 could account for only approximately two per cent of the potency of serum whereas the components of Solution 2 were even less active. A mixture of substances in both solutions (Solutions 1 and 2) was considerably more effective but could account for only approximately 13 per cent of the potency of total serum. The potency of the latter mixture compared favorably with the potency of the UM-2 ultrafiltrate described earlier.

Of the substances tested thus far in this study, the proteins made the largest contribution to the potency of total serum. However, the failure of the mixture of known proteins to account for the entire potency of serum suggested that the

TABLE II

*Composition of solutions prepared for measuring potency of substances of low molecular weight found in human plasma.**

Solution 1		Solution 2	
Compounds included	Upper limit of normal conc. in human plasma (mg/100 ml)	Compounds included	Upper limit of normal conc. in human plasma (mg/100 ml)
Alanine	3.7	Bilirubin	1.4
Alpha-aminobutyric acid	0.4	Citric acid	3.0
Arginine	1.9	Creatine	0.9
Asparagine	0.7	Creatinine	2.0
Aspartic acid	0.07	Fructose	8.0
Cysteine and cystine	1.3	Glucose	90.0
Glutamic acid	1.2	Alpha-ketoglutaric acid	1.0
Glutamine	12.0	Lactic acid	17.0
Glycine	1.7	Malic acid	0.9
Histidine	1.5	Ornithine	0.8
Isoleucine	1.3	Pyruvic acid	2.0
Leucine	2.3	Succinic acid	0.6
Lysine	3.0	Uric acid	6.0
Methionine	0.4		
Phenylalanine	1.0		
Proline	3.3		
Serine	1.2		
Taurine	0.8		
Threonine	1.7		
Tryptophan	1.2		
Tyrosine	1.5		
Urea	30.0		
Valine	3.7		

* Compounds and concentrations given by White, Handler and Smith (1964, p. 628). Stock solutions were prepared with all constituents present at concentrations ten-fold the values shown in table.

presence of certain substances of low molecular weight, together with the proteins, might be necessary to account for the total activity of serum itself. To test this possibility a mixture containing the six dialyzed proteins described earlier was combined with a mixture of Solution 1 and Solution 2 for testing. Figure 2 shows that this mixture provided a solution that was fully as active as total serum. The fact that this mixture was seemingly somewhat more potent than serum itself was probably due to the fact that each substance in the mixture was added at a concentration proportionate to the normal upper limit reported for plasma. Although this practice provided solutions in which the various constituents of serum occurred at their proper relative concentrations, this practice logically resulted in the incorporation of certain constituents at concentrations higher than those which actually existed in the samples of total serum that were used as a standard.

Bioassays of individual substances of low molecular weight

In conjunction with the tests of mixtures of substances of low molecular weight found in human serum, several of these same substances were assayed individually over a range of concentrations of 10^{-2} to 10^{-4} M. In addition to glycine (ED_{50} *ca.* 5×10^{-3} M), two other substances were found to be moderately strong response-inducers when tested alone. These substances were L-glutamic acid (ED_{50} *ca.* 10^{-2} M) and taurine (ED_{50} *ca.* 4×10^{-3} M). Individually, the activities of glycine, glutamic acid, and taurine accounted for only 0.01 to 0.08 per cent of the potency of total serum (see Fig. 2). Slight activity was also obtained with citric acid, D-glucose, and betaine (not reported to be a constituent of human plasma). However, the ED_{50}, if any, for each of these latter substances was greater than 10^{-2} M. A mixture of glycine, glutamic acid, taurine, citric acid, and glucose was prepared with each substance present at the same relative concentration as in plasma (see Table II). This mixture could account for only approximately one per cent of the potency of serum itself and was slightly less active than the mixture of substances in Solution 1 described earlier.

None of the following substances were response-inducers over the range of concentrations indicated as follows: 10^{-2} to 10^{-3} M, N-acetylglucosamine, N-acetylglycine, glycerol, lactic acid, proline, and trimethylamine oxide; 10^{-3} to 10^{-4} M, glutathione, glycerophosphorylcholine, octopine, octopinic acid; 10^{-4} to 10^{-5} M, cyclic adenosine monophosphate, L-3,4 dihydroxyphenylalanine (DOPA), L-epinephrine, melatonin, and serotonin. Of the substances listed above, only lactic acid and proline are considered to be among the major constituents of normal human plasma.

Preliminary tests with body fluids and extracts from other sources

A series of other body fluids and extracts were assayed before and after fractionation by ultrafiltration. In two of the preparations substances of greater than *ca.* 1000 molecular weight (presumably proteins) were the major stimulants, whereas smaller molecules were the major stimulants in the other three preparations. With oyster mantle fluid and with coquina extract, the retentates were fully as active as the original preparations. The ultrafiltrates of both were significantly less active. With crab serum, lobster extract and shrimp extract, the major stimulants were present in the ultrafiltrates which in each case were not significantly less active than the original preparations. The retentates of each of these latter preparations were significantly less active.

Discussion

Palaemonetes pugio proved to be an excellent test animal for studies of chemoreception. This small shrimp abounds near the shoreline of the estuarine habitat near our laboratory. It is easy to maintain in dense populations with the reservation that considerable cannibalism occurs especially upon freshly molted individuals. The bioassay procedure that was described is both convenient and reliable. This procedure allows the investigator to quickly recognize response-inducing solutions and to compare in a quantitative manner the relative potencies of closely related solutions.

Our results clearly show that the effectiveness of human serum as a chemosensory stimulant in *P. pugio* is due to a mixture of substances rather than to a single major stimulant. This mixture of substances includes both proteins and a group of components of low molecular weight. Our finding that proteins make a major contribution to the potency of this mixture provides an important corollary to several other recent reports on the nature of feeding stimulants in other groups of marine invertebrates. Gurin and Carr (1971) showed that in the gastropod, *Nassarius obsoletus* (phylum Mollusca), stimulation of the probosis search reaction by human serum and oyster fluid could be ascribed primarily to very low concentrations of specific proteins. In human serum the major stimulant was serum albumin whereas in oyster fluid it was a large glycoprotein. Additional evidence that proteins present in other biological fluids and extracts serve as important feeding stimulants in *N. obsoletus* was provided by Carr, Hall and Gurin (1974). Mangum and Cox (1971) reported evidence that a glycoprotein in a mollusk extract made a significant contribution to stimulation of a feeding response in the polychaete, *Diopatra cuprea* (phylum Annelida). Heeb (1973) found that protein fractions isolated from two mollusk extracts made an important contribution to stimulation of feeding behavior in the starfish, *Asterias forbesi* (phylum Echinodermata).

In *P. pugio*, the fact that proteins make an important contribution to the stimulatory capacity of human serum does not justify the assumption that proteins make an important contribution to the activity of all preparations that stimulate feeding behavior in this animal. Preliminary tests with other body fluids and with extracts showed that the nature of the major feeding stimulants are apt to vary in preparations obtained from different sources. In crab serum and in extracts of shrimp and lobster it is clear that the major stimulants are not proteins but are substances of reasonably low molecular weight. However, the results of our tests with the common amino acids, amines, organic acids, and carbohydrates suggest strongly that these substances alone will not prove to be the major stimulants since the concentrations at which they occur in these latter preparations are inadequate to account for the potencies of the crab serum or the extracts themselves. Additional studies of the stimulants in these preparations are in progress.

The possession of chemoreceptors sensitive to amino acids and/or other nitrogenous compounds of low molecular weight is characteristic of effectively all marine arthropods that have been studied to date using either behavioral procedures or electrophysiological techniques. In the current study with *P. pugio,* taurine (*ca.* 0.004 M), glycine (0.005 M), L-glutamic acid (*ca.* 0.01 M), and betaine (> 0.01 M) were found to be response-inducers when tested alone. It is of interest that sufficient concentrations of one or more of the above substances have also been reported to induce feeding behavior and/or elicit chemoreceptor activity in a variety of other marine arthropods. Case and Gwilliam (1961) found that dactyl receptors of the crab, *Carcinides maenas,* were especially sensitive to L-glutamic acid at concentrations as low as 0.00005 M. Moreover, the application of either 0.001 M glutamic acid or of *Mytilus* extract to the chelae stimulated the beginning of feeding movements. However, no report of the concentration of glutamic acid in the extract was given. Laverack (1963) found dactyl receptors of *C. maenas* sensitive to betaine (0.1 to 0.001 M). Laverack (1964) later reported the presence of antennular receptors of the spiny lobster, *Panulirus argus,* that were sensitive to 0.1 M betaine, glycine, and L-glutamic acid. Levandowski and Hodgson (1965) also reported that antennular and dactyl receptors of *P. argus* were sensitive to betaine and L-glutamic acid but less sensitive to glycine. The horseshoe crab, *Limulus,* possesses gnathobase receptors sensitive to 0.5 M glycine, glutamic acid and taurine (Barber and Hayes, 1963). However, clam extracts were noted by the latter workers to elicit responses stronger than any given to amino acids when tested either alone or as mixtures. Case (1964) studied thoroughly certain properties of dactyl chemoreceptors of two crabs, *Cancer antennarius* and *C. productus.* Response magnitudes to various substances were compared to those evoked by 0.05 M glycine. Included among the substances with response magnitudes greater than glycine were taurine, L-glutamic acid and betaine. Crisp (1967) reported that L-glutamic acid and taurine (*ca.* 0.00002 M) were the most effective substances he tested for inducing feeding movements of cirri in two species of stalked barnacles. Betaine (*ca.* 0.0002 M) and glycine (*ca.* 0.002 M) were also active. Two species of commensal shrimp of the genus *Betaeus* were found by Ache and Case (1969) to possess antennular receptors sensitive to glutamate and glycine (*ca.* 0.1 M). McLeese (1970) found glutamic acid (*ca.* 0.7 ppm) to be among the most effective inducers of a feeding response in the lobster, *Homarus americanus.* McLeese pointed out that although several amino acids and other substances induced feeding behavior either alone or in mixtures, none of the substances or mixtures were as stimulatory as extracts of cod, shrimp or lobster muscle. In summary one must conclude that although an array of marine arthropods have been shown to have chemoreceptors capable of detecting certain amino acids and related substances, no studies have shown that these substances can account for the major portion of the stimulatory activity of a single tissue extract or biological fluid.

Copeland (1923) reported that *Palaemonetes vulgaris* could find fish meat buried in sand or in a tube covered with cloth. His early studies showed that chemical agents emanating from food served to attract the shrimp to this food. The age-old practices of attracting crabs and lobsters into baited traps and of chumming the water for shrimp are examples of the potential that exists for exploiting the chemical sense of marine invertebrates as a means of increasing the harvest of seafood. Technological advances in this area are likely to be forthcoming as soon as the chemical nature of the principal attractants in "baits" and "chum" are determined. Such determinations should be enhanced greatly by the development and utilization of quantitative bioassay procedures which permit the investigator to compare directly the potencies of various baits as well as the potencies of the specific chemical substances that they contain.

SUMMARY

1. A bioassay procedure is described for studying a chemically mediated feeding response in the shrimp, *Palaemonetes pugio*. The procedure involves the attraction of shrimp to a small target in compartmented plastic boxes.

2. Bioassays of purified components of human serum showed that the response of shrimp to serum is due to a mixture of substances including both proteins and substances of low molecular weight.

3. A dialyzed mixture containing six types of serum proteins accounted for approximately one-fourth of the potency of serum. Although all of the proteins possessed a certain degree of activity, low density lipoprotein and the alpha$_1$-globulins were individually the most active components of the mixture.

4. A mixture containing the 37 major low molecular weight organic constituents of serum accounted for only approximately one-eighth of the potency of serum. Glycine, taurine and glutamic acid were the most active constituents of this mixture that were tested individually.

5. A mixture containing the six types of serum proteins together with the 37 low molecular weight constituents was fully as active as the total serum.

6. Ultrafiltration of body fluids or extracts from coquina, crab, lobster, oyster and shrimp showed that in some preparations the major stimulants were large molecules (greater than *ca.* 1000 MW) whereas in others they were small molecules.

LITERATURE CITED

Ache, B., and J. Case, 1969. An analysis of antennular chemoreception in two commensal shrimps of the genus *Betaeus*. *Physiol. Zool.*, **42**: 361–371.

Barber, S. B., and W. P. Hayes, 1963. Properties of *Limulus* chemoreceptors. *Proc. XVI Int. Congr. Zool.*, **3**: 76–78.

Carr, W. E. S., 1967. Chemoreception in the mud snail, *Nassarius obsoletus*. I. Properties of stimulatory substances extracted from shrimp. *Biol. Bull.*, **133**: 90–105.

Carr, W. E. S., E. R. Hall, and S. Gurin, 1974. Chemoreception and the role of proteins: a comparative study. *Comp. Biochem. Physiol.*, **47A**: 559–566.

Case, J., and G. F. Gwilliam, 1961. Amino acid sensitivity of the dactyl chemoreceptors of *Carcinides maenas*. *Biol. Bull.*, **121**: 449–455.

Case, J., 1964. Properties of dactyl chemoreceptors of *Cancer antennarius* Stimpson and *C. productus* Randall. *Biol. Bull.*, **127**: 428:446.

Copeland, M., 1923. The chemical sense of *Palaemonetes vulgaris*. *Anat. Rec.*, **24**: 394.

Crisp, D. J., 1967. Chemoreception in cirripedes. *Biol. Bull.*, **133**: 128–140.

Daum, R. J., and C. Givens, 1963. *Potency probit analysis.* U .S. Dept. of Agriculture, Biometrical Services, Beltsville, Md., 23 pp.

Davenport, D., 1966. Analysis of behavior in symbiosis. Pages 381–429 in S. M. Hendry, Ed., *Symbiosis*. Academic Press, N. Y.

Gurin, S., and W. E. S. Carr, 1971. Chemoreception in *Nassarius obsoletus:* the role of specific stimulatory proteins. *Science*, **174**: 293–295.

HEEB, M. A., 1973. Large molecules and chemical control of feeding behavior in the starfish *Asterias forbesi, Helgolander Wiss. Meeresunters,* **24:** 425–435.

KITTREDGE, J. S., M. TERRY, AND F. T. TAKAHASHI, 1971. Sex pheromone activity of the molting hormone, crusteedysone, on male crabs. *Fish. Bull., Nat. Mar. Fish. Serv.,* **69**: 337–343.

KITTREDGE, J. S., F. T. TAKAHASHI, J. LINDSEY, AND R. LASKER, 1974. Chemical signals in the sea: marine allelochemics and evolution. *Fish. Bull., Nat. Mar. Fish. Serv.,* **72**: 1–11.

LAVERACK, M. S., 1963. Aspects of chemoreception in crustacea. *Comp. Biochem. Physiol.,* **8**: 141–151.

LAVERACK, M. S., 1964. The antennular sense organs of *Panulirus argus. Comp. Biochem. Physiol.,* **13**: 301–321.

LEVANDOWSKI, M., AND E. S. HODGSON, 1965. Amino acid and amine receptors of lobsters. *Comp. Biochem. Physiol.,* **16**: 159-161.

LINDSTEDT, K. J., 1971. Chemical control of feeding behavior. *Comp. Biochem. Physiol.,* **39A**: 553–581.

MANGUM, C. P., AND C. D. COX, 1971. Analysis of the feeding response in the onyphid polychaete *Diopatra cuprea* (Bosc). *Biol. Bull.,* **140**: 215–229.

McLEESE, D. W., 1970. Detection of dissolved substances by the American lobster (*Homarus americanus*) and olfactory attraction between lobsters. *J. Fish. Res. Bd. Canada,* **27**: 1371–1378.

RYAN, E. R., 1966. Pheromone: evidence in a decapod crustacean. *Science,* **151**: 340–341.

SCANU, A. M., AND C. WISDOM, 1972. Serum lipoproteins structure and functions. *Ann. Rev. Biochem.,* **41**: 703–730.

WHITE, A., P. HANDLER, AND E. L. SMITH, 1964. *Principles of biochemistry,* (3rd ed.) McGraw-Hill Book Co., N. Y., 1106 pp.

Reference: *Biol. Bull.*, **148**: 393–401. (June, 1975)

ENVIRONMENTAL REGULATION OF THE ANNUAL REPRODUCTIVE SEASON OF *STRONGYLOCENTROTUS PURPURATUS* (STIMPSON)

ROGER C. COCHRAN AND FRANZ ENGELMANN

Department of Biology, University of California, Los Angeles, California

Along the length of the Pacific Coast from Alaska to Cedros Island, the gonads of *Strongylocentrotus purpuratus* contain numerous mature gametes during the winter months (Lasker and Giese, 1954; Bennett and Giese, 1955; Holland and Giese, 1965; Boolootian, 1966; Chatlynne, 1969; Cochran and Engelmann, 1972; Gonor, 1973a, 1973b). Following this season, the gonads are depleted of gametes, and nutritional reserves appear to be accumulated (Holland and Giese, 1965; Gonor, 1973a). In the fall and early winter months, more and more gametes are produced and accumulate. The synchrony of the annual reproductive cycles of different populations of *S. purpuratus* has led to suggestions that they are regulated by environmental factors (Bennett and Giese, 1955; Boolootian, 1963; Chatlynne, 1969; Gonor, 1973a; Giese and Pearse, 1974).

Boolootian (1963) reported a proliferation of spermatogonia under the stimulus of long-day photoperiod (14L:10D), while short-day (6L:18D) presumably induced complete maturation of sperm within three weeks. Others (Bennett and Giese, 1955; Chatlynne, 1969) have suggested that temperature, or food availability (Gonor, 1973a) may be the agents regulating the reproductive cycle. On the other hand, it has been reported that a laboratory population of *S. purpuratus* in constant temperature (15° ± 1° C) and light, approximating natural conditions, remained reproductively synchronous with the field populations over a three year period (Boolootian, 1964). This latter information raises the possibility of an endogenous near annual rhythm of reproduction, independent of environmental factors (Boolootian, 1966).

In conjunction with research on echinoid spawning factors (Cochran and Engelmann, 1972), the current study was undertaken to analyze the effects of photoperiod and/or temperature on the onset and termination of the reproductive activity of a Southern California subtidal population of *S. purpuratus*. Subtidal animals were chosen because changes in environmental conditions, such as temperature, salinity, and food availability, are generally more gradual than in the intertidal zones.

Materials and Methods

All specimens of *S. purpuratus* were collected at Flat Rock Point on the Palos Verdes Peninsula (Los Angeles, California) at depths of two to eight meters using SCUBA and skin-diving equipment. The animals were transported in burlap bags to holding tanks within one hour of collection. Approximately $\frac{1}{3}$ of the animals collected in this manner die within the first two weeks, but there is not much subsequent attrition.

The following arrangement was used for the photoperiod experiments: four 150-liter tanks were arranged in two tiers of two tanks each. All external light was excluded from the tanks by means of heavy, opaque, rubberized curtains. The top tier was insulated from the light of the bottom tier as well. Light was provided by two 100-watt tungsten-filament bulbs over each tank, and controlled by timer clocks. The light cycle for the top tier was independent from the cycle of the bottom tier. The artificial light:dark cycles used here corresponded to the photoperiods of the summer and winter solstices at Flat Rock Point, 14L:10D and 10L:14D respectively. Sea water in the tanks was obtained from a general 40,000 liter sea water system, which continuously cooled, filtered, and aerated the water. Additional aeration was provided by two airstones in each tank. Fresh, brown algae (*Egregia* sp.) was given weekly as food for the sea urchins.

Animals were considered reproductively active if mature gametes, discernible with the unaided eye, oozed from the severed gonads (Cochran and Engelmann, 1972). Reproductiviely active and inactive sea urchins were exposed to either long- or short-day photoperiods for up to 90 days. Animals used in each experiment were collected not earlier than two days prior to the initiation of an experiment. At the end of all experiments, sea urchins were cut open to ascertain their reproductive condition. Animals taken from the ocean at the time these experiments were terminated were used for comparison.

For the temperature experiments, the water temperature in one tank was modified by a Lauda thermostat refrigeration and pump unit. Low temperature (13° C) was maintained for up to 90 days, and high temperature (19° C) for 30 days. These temperatures approximate the minimum and maximum ocean temperatures encountered by the subtidal sea urchin population at Flat Rock Point. The temperature of the water of the second tank in the same tier was that of the general sea water system (average 14° C). Animals were sampled from time to time to ascertain their reproductive condition.

RESULTS

Photoperiod

In the first experiment, begun September 19, 1970, 30 reproductively inactive animals were placed in each of the four tanks. The photoperiod of the top tier was 10L:14D, and that of the bottom tier was 14L:10D. The experiment was terminated six weeks later on November 1, 1970. A subsequent experiment was begun November 8, 1970 with 50 animals (27% reproductively active) per tank under the same photoperiod regimes. This experiment was terminated 13 weeks later on February 14, 1971. The water temperature during these and following photoperiod experiments averaged 13° C, with a range from 12 to 14.5° C. As is seen (Table I), neither long- (14L:10D) nor short-day (10L:14D) photoperiods affected the onset of reproductive activity, either accelerating or inhibiting the appearance of spawnable gametes. In none of the experimental series was the reproductive activity significantly different from that of the subtidal field populations. A marginally significant difference was, however, found between the long- and short-day photoperiod populations in the experiments ending November 1,

Table I

The effect of long- and short-day photoperiod on onset or termination of spawning capability. Comparison between the two laboratory populations (P), and between laboratory and field populations (P′)(Chi square).

Photoperiod	Duration of experiment (weeks)	Laboratory animals		Ocean animals	
		Active/total	*P*	Active/total	*P′*
10L:14D	6	17/46	<2.5%	8/30	<40%
14L:10D	6	5/35			<30%
10L:14D	13	73/76	<20%	20/20	<50%
14L:10D	13	57/63			<20%

1970. No difference was found between the long- and short-day photoperiod populations of the second experiment ending February 14, 1971.

A second series of experiments was begun on July 14, 1971, when the animals were in their early reproductively inactive phase (Cochran and Engelmann, 1972). Fifty sea urchins were placed in each of the four tanks, and the photoperiods of the two tiers were left the same as in the previous experiments. Animals from the top tier (10L:14D) were sacrificed 10 weeks later (October 1, 1971). The proportion of reproductively active animals in these short-day populations was not significantly different from that of the field population (Table II). Obviously the 10 weeks of short-day treatment did not induce early reproductive activity. New animals from the field were placed in the top tier (50 per tank) at this time, and the photoperiod was changed to 14L:10D, the same as the bottom tier. All sea urchins from the four tanks were then sacrificed on December 12, 1971. The reproductive activity of animals kept for only 10 weeks under long-day photoperiod was not significantly different from that of animals kept for 20 weeks under

Table II

Effects of extended long- and short-day photoperiods on the reproductive activity of the purple sea urchin. P values (chi square) are obtained for a comparison between the laboratory and ocean populations.

Photoperiod	Duration of experiment (weeks)	Laboratory animals Active/total	Ocean animals at termination Active/total	*P*
10L:14D	10	3/21	0/20	<10%
10L:14D	10	4/28		<10%
14L:10D	10	32/36	20/20	<20%
14L:10D	10	34/38		<20%
14L:10D	20	20/21		<50%
14L:10D	20	21/23		<20%

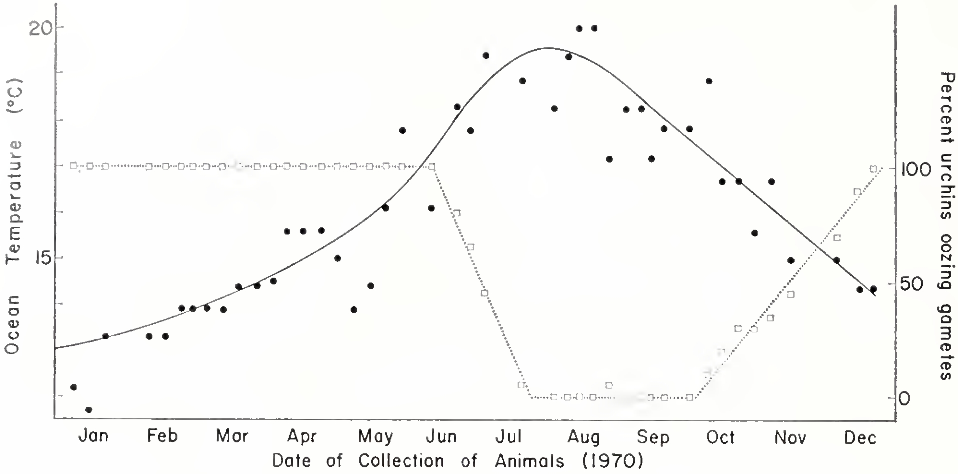


FIGURE 1. Annual reproductive cycle of *S. purpuratus*, as measured by the percentage of animals that released gametes when cut open (dotted curve) superimposed on the seasonal variation in ocean temperature (solid line) at the same location. Subtidal ocean temperatures were taken on the dates of collection.

similar conditions. The reproductive activity of the two laboratory populations did not differ from that of the field population. Thus long-day photoperiod (14L:10D) did not inhibit the onset of reproductive activity. It should be mentioned here that the water temperature (13° C) in the holding tanks was markedly lower than that in the field throughout most of this period (Figure 1).

Temperature

It became apparent during these studies that the annual reproductive cycle of *S. purpuratus* from Palos Verdes is inversely correlated with the annual fluctuation of ocean temperature at the same location (Figure 1). A rise of water temperature above 17° C coincides with the loss of the ability to spawn. A series of experiments were thus undertaken in order to ascertain whether temperature is indeed the environmental cue for the regulation of the reproductive season of this population.

In an experiment begun May 1, 1972, almost all animals maintained at 13° C (the normal winter temperature) and a photoperiod of 14L:10D remained reproductively active for nearly two months beyond the date (late June) when animals in the field had become reproductively inactive (Table III). During this experiment, for a period of 10 days in late July, the temperature of the general sea water system accidently rose to 17.5° C, while that of the regulated tank reached 16.5° C. Prior to, and after this period, the temperature of the general sea water system averaged 14.5° C (13.5° to 15° C) and that of the regulated tank 13° C (12.5° to 13.5° C). Before this short term rise in temperature, there had been no significant difference between the proportions of reproductively active animals in the two laboratory populations. The proportion of reproductively active animals in

TABLE III

Effects of low temperature on reproductive activity in S. purpuratus.

	Elapsed time (weeks)	Experimental tanks			Sea water system			Ocean		
		Temp. (° C)	Sea urchins Active/total	%	Temp. (° C)	Sea urchins Active/total	%	Temp. (° C)	Sea urchins Active/total	%
At initiation	0	13			15			13.9	30/30	100
First sampling	7	13	9/10	90	14	10/10	100	17.8	2/20	10
Second sampling	10	13.5	9/10	90	14	8/10	80	18.9	2/20	10
Third sampling	12	16.5*	10/10	100	17.5*	5/10	50	20.5	0/20	0
Fourth sampling	14	12.5	13/14	93	14	6/17	33	20.5	0/20	0

* For a period of 10 days in late July the temperature of the general sea water system reached 17.5° C, while that of the experimental tanks was 16.5° C.

both of these laboratory populations was significantly different from that of the ocean population ($P < 0.5\%$). The rise in temperature to 17.5° C caused a significant ($P < 1\%$) reduction in the proportion of reproductively active animals compared to the other laboratory animals at 16.5° C. This difference in reproductive activity between the two laboratory populations was even more significant ($P < 0.5\%$) at the time the experiment was terminated. These results indeed suggest that reproductive activity in this species of sea urchin is turned off by an elevated temperature.

In a further effort to test the hypothesis that warm temperature terminates reproductive activity, 50 animals were placed in each of two adjacent tanks (water temperature 12.5° C) during the height of the reproductive season (February 11, 1973). In the course of 11 days the water temperature in one tank was raised to 19° C. Three weeks later, nearly all surviving animals had either spawned or resorbed their gametes (Table IV). During this entire period the animals were kept in short-day photoperiod (10L:14D).

The converse experiment, *i.e.*, induction of reproductive activity by cool water temperature, was started on September 15, 1972 with 50 animals in each tank. After eight weeks of low temperature (13° C), the proportion of reproductively active animals in either tank was not significantly different from that of the field population (Table V). Also, an experiment started in July of 1970 to test the effects of short-day photoperiod was performed at 13° C for 10 weeks (Table II).

TABLE IV

Reproductive activity of S. purpuratus *as affected by elevated temperature.*

	Experimental tanks			Sea water system			Ocean		
	Temp. (° C)	Sea urchins Active/total	%	Temp. (° C)	Sea urchins Active/total	%	Temp. (° C)	Sea urchins Active/total	%
At initiation	17			12.5			13.3	20/20	100
At termination	19	1/20	5	12.5	42/43	98	13.3	40/40	100

TABLE V

Effect of reduced temperature on inducing reproductive activity in S. purpuratus *during the late inactive season.*

	Elapsed time (weeks)	Experimental tanks			Sea water system			Ocean		
		Temp. (° C)	Sea urchins Active/total	%	Temp. (° C)	Sea urchins Active/total	%	Temp. (° C)	Sea urchins Active/total	%
At initiation	0	13			14			20.5	0/20	0
First sampling	7	12.5	0/20	0	14	0/20	0	16.1	3/20	15
Second sampling	8	13.5	2/20	10	15	3/20	15	15.1	5/20	25

Here too, the proportion of reproductively active animals was not significantly different from that of the field population. Thus, under these experimental laboratory conditions, the onset of spawning capability was not hastened.

Observations made in the field study area during 1973 tend to support the concept of temperature-regulated reproductive activity in *S. purpuratus*. Animals collected during March and April of 1973 were reproductively inactive (an unusual phenomenon for this season) and the gonads had the spent appearance normally observed at the end of the reproductive season. However, subsequent to May 11, all of the sea urchins collected had copious amounts of gametes. This renewal of reproductive activity, following a "spawn out" (possibly caused by low-salinity shock or turbulence from an unusually high number of rain storms passing through the area) occurred while the field population of sea urchins was still experiencing low temperatures. Even through the summer months of 1973, 10% of the sea urchin population retained spawning capability, which correlated with an unusually cold summer ocean temperature.

DISCUSSION

The designation, reproductive season, breeding season, or reproductive period is generally based on the gonad index, presence of numerous mature gametes, or size- and stage-frequency analysis of oocytes in *S. purpuratus* (Boolootian, 1966). Each of these methods, however useful, has limitations (Gonor, 1972, 1973a; Giese and Pearse, 1974). The first appearance of numerous mature gametes, coupled with a high gonad index, signals the onset of the reproductive season. The actual frequency of spawning by *S. purpuratus* field populations during this period is unknown. Studies on gametogenesis reveal a continuous maturation of gametes during the reproductive season which makes possible several spawning events (Holland and Giese, 1965; Chatlynne, 1969). The spawned out appearance, which marks the end of the reproductive season, is caused by released or resorbed gametes not being completely replaced by new ones (Chatlynne, 1969; Gonor, 1973a, 1973b).

As many observations suggest (Bennett and Giese, 1955; Boolootian, 1966; Gonor, 1973a), the reproductive season of *S. purpuratus* is probably controlled by environmental factors. Figure 1 illustrates the annual reproductive cycle for

1970 in the Palos Verdes subtidal population. A drop in spawning capability occurs around the summer solstice, while full reproductive activity is achieved a couple of weeks before the winter solstice. This seemingly agrees with the hypothesis that reproductive activity of *S. purpuratus* is affected by changing photoperiods (Boolootian, 1963). However, the main body of data given in this paper (Tables I, II) indicates that short-day photoperiod (10L:14D) does not induce the onset of reproductive activity. Only 14% of the animals in populations kept under short-day photoperiod (10L:14D) for 10 weeks during the summer became reproductively active, and this was not significantly different from the reproductive activity of the ocean population (Table II). One might expect a much greater proportion of reproductively active animals since the maturation and growth of ova from primary oocytes can be completed in approximately this time (Pearse, Clark, Leighton, Mitchell, and North, 1970), and the maturation of sperm under short-day photoperiod (6L:18D) takes only three weeks (Boolootian, 1963). Conversely, the reproductive activity of animals kept under long-day photoperiod (14L:10D) from July 19 until December 12, 1971 (the beginning of the natural reproductive season) was not significantly different from the subtidal population. Thus the onset of reproductive activity was not retarded by the lack of short-day photoperiod. The marginally significant difference ($P < 2.5\%$) in the proportion of reproductively active animals between two experimental populations kept under short- and long-day photoperiods respectively while at 13.5° C (Table I) cannot be overlooked. The preponderance of evidence, however, suggests that photoperiod has no direct influence on initiation or termination of reproductive activity.

The annual oscillation in ocean temperature has been suggested as a possible cue for synchronizing an endogenous reproductive rhythm in *S. purpuratus* (Bennett and Giese, 1955; Chatlynne, 1969). In Figure 1, ocean temperature and reproductive activity of *S. purpuratus* during 1970 are superimposed. In the laboratory, reproductive activity could be maintained by low temperature (13° C) (Table III), and terminated by warm temperature (19° C) (Table IV). These findings are in agreement with the results obtained by Dr. John Pearse on the same species (personal communication). He has demonstrated that sea urchins kept for one month at 7° C or 14° C retained copious gametes in the gonads, while those at 21° C resorbed most of their gametes. Thus, there is substantial evidence that the reproductive season of *S. purpuratus* is terminated by increased temperature.

The untimely loss of reproductive activity during March and April of 1973 (possibly caused by low-salinity shock or turbulence from an unusually high number of rain storms passing through the area) occurred while the ocean temperature was less than 14.5° C. Normally in the Palos Verdes area, a population "spawn out" does not occur until mid-June, accompanied by a rise in ocean temperature above 17° C. Perhaps this rise in temperature induces spawning (Giese and Pearse, 1974), and does not allow a continued gamete maturation. During late spring of 1973, however, the sea urchins regained full reproductive activity within a few weeks, an indication that the absence of gametes was due to frequent spawning and that gamete maturation had not abated. These field observations thus support the hypothesis that reproductive activity is halted by increased ocean temperature.

While the control of the termination of reproductive activity has been elucidated in at least one case, the environmental factor(s) regulating the onset of reproductive activity has yet to be determined. In the laboratory, neither short-day photoperiod (10L:14D) nor low temperature (13° C) accelerated the onset of reproductive activity in this sea urchin (Tables II, IV). This raises the possibility that the control of the onset of reproductive activity is more complex than we originally suspected. For example, the duration of the inactive period that follows the reproductive season may be a function of the time necessary to acquire sufficient energy reserves to generate mature gametes. In support of this hypothesis one can cite the northern intertidal populations of *S. purpuratus,* which depend upon seasonally varying amounts of algal drift (Gonor, 1973a). This population is reproductively inactive longer than southern, subtidal sea urchins, which continuously obtain their nutrition by grazing on algae-encrusted rocks (Pearse, Clark, Leighton, Mitchell, and North, 1970; Leighton, 1971).

Although ocean temperature plays a role in the reproductive activity of a Southern California subtidal population of *S. purpuratus,* it may not be the only cue for other populations. Northern, subtidal sea urchins, not exposed to the high temperatures of southern latitudes, may rely on a different critical temperature. Also, intertidal populations, both north and south, experience frequent fluctuations in temperature, salinity, and food availability and thus might be expected to cue on a more predictable environmental factor, such as photoperiod. However, at present no information is available to show that this is indeed the case.

Some of the facilities used in this study were purchased with funds obtained through NSF Grant GB 14965 (Engelmann). We thank Drs. J. S. Pearse and J. T. Enright for valuable suggestions and criticisms during the preparation of the typescript; any conclusions drawn are entirely our own.

SUMMARY

1. The annual reproductive cycle of the Palos Verdes (Southern California) subtidal population of *S. purpuratus* correlates with seasonal changes in ocean temperature: a rise of temperature above 17° C (June) coincides with cessation of gamete production and storage.

2. Sea urchins maintained at low temperature (13° C) retained gametes nearly two months (as long as they were observed) beyond the date when the field population had "spawned out."

3. At a time when the field population was reproductively active, the spawning capability of a laboratory population was terminated by three weeks of warm water temperature (19° C).

4. The spawning capability of animals kept at 13° C was not terminated by long-day photoperiod (14L:10D).

5. The onset of reproductive activity in animals started during the summer months could not be accelerated experimentally by 10 weeks of low temperature (13° C).

6. In laboratory animals maintained at 13° C, the onset of reproductive activity was neither enhanced by short-day (10L:14D), nor retarded by long-day (14L:10D) photoperiod.

LITERATURE CITED

BENNETT, J., AND A. C. GIESE, 1955. The annual reproductive and nutritional cycles of two western sea urchins. *Biol. Bull.,* **109**: 226–237.

BOOLOOTIAN, R. A., 1963. Response of the testes of purple sea urchins to variations in temperature and light. *Nature,* **197**: 403.

BOOLOOTIAN, R. A., 1964. Die Bedeutung abiotischer Faktoren für die Gonadenentwicklung und Fortpflanzung mariner Evertebraten. *Helgoländer wiss. Meeresuntersuch.,* **10**: 118–139.

BOOLOOTIAN, R. A., 1966. Reproductive physiology. Pages 561–613 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Wiley and Sons, New York.

CHATLYNNE, L. G., 1969. A histochemical study of oogenesis in the sea urchin, *Strongylocentrotus purpuratus. Biol. Bull.,* **136**: 167–184.

COCHRAN, R. C., AND F. ENGELMANN, 1972. Echinoid spawning induced by a radial nerve factor. *Science,* **178**: 423–424.

GIESE, A. C., AND J. S. PEARSE, 1974. Introduction: general principles. Pages 1–49, in A. C. Giese and J. S. Pearse, Eds., *Reproduction of Marine Invertebrates. Vol. I.* Academic Press, New York.

GONOR, J. J., 1972. Gonad growth in the sea urchin, *Strongylocentrotus purpuratus* (Stimpson) (Echinodermata: Echinoidea) and the assumptions of gonad index methods. *J. Exp. Mar. Biol. Ecol.,* **10**: 89–103.

GONOR, J. J., 1973a. Reproductive cycles in Oregon populations of the echinoid, *Strongylocentrotus purpuratus* (Stimpson). I. Annual gonad growth and ovarian gametogenic cycles. *J. Exp. Mar. Biol. Ecol.,* **12**: 45–64.

GONOR, J. J., 1973b. Reproductive cycle in Oregon populations of the echinoid, *Strongylocentrotus purpuratus* (Stimpson). II. Seasonal changes in oocyte growth and in abundance of gametogenic stages in the ovary. *J. Exp. Mar. Biol. Ecol.,* **12**: 65–78.

HOLLAND, N. D., AND A. C. GIESE, 1965. An autoradiographic investigation of the gonads of the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.,* **133**: 578–590.

LASKER, R., AND A. C. GIESE, 1954. Nutrition of the sea urchin, *Strongylocentrotus purpuratus. Biol. Bull.,* **106**: 328–340.

LEIGHTON, D. L., 1971. Grazing activities of benthic invertebrates in Southern California kelp beds. *Nova Hedwigia, Beiheft* **32**: 421–453.

PEARSE, J. S., M. E. CLARK, D. L. LEIGHTON, C. T. MITCHELL, AND W. J. NORTH, 1970. Marine waste disposal and sea urchin ecology. Pages 1–93 in W. J. North, Ed., *Kelp Habitat Improvement Project, Annual Report 1969–1970.* California Institute of Technology, Pasadena.

Reference: *Biol. Bull.*, **148**: 402–415. (June, 1975)

EFFECTS OF PHOTOPERIOD AND TEMPERATURE ON GONADAL ACTIVITY IN THE CYPRINID TELEOST, *NOTEMIGONUS CRYSOLEUCAS*

VICTOR L. DE VLAMING

Department of Biology, Marquette University, Milwaukee, Wisconsin 53233

Recent literature reviews (de Vlaming, 1972a, 1974) indicate that there is considerable variation among teleost fishes with regard to environmental control of reproductive cycling. Photoperiod may exert the dominant regulatory role of sexual cycles in the salmonids and gasterosteids. Temperature appears to be extremely important in regulating reproductive rhythms in the cyprinodontiform fishes.

Daylength changes in combination with various temperatures seem to be important in controlling reproduction in the cyprinid and centrarchid fishes (*cf.* de Vlaming, 1972a, 1974). Champy (1923) suggested that warm temperature treatment in late autumn stimulates the completion of spermatogenesis in the cyprinid, *Phoxinis laevis;* the potential role of photoperiod in gonadal maturation or the possible seasonal change in response to temperature were not discussed. A later study (Bullough, 1939) with this species indicated that high temperatures promote the early phases of spermatogenesis and oogenesis, regardless of photoperiod. Bullough (1939) implied that long photoperiods are required for the final stages of gonadal maturation in *Phoxinus* captured in autumn. In a later study, Bullough (1940) reported that *Phoxinus* maintained under natural temperature conditions, but on a short photoperiod from autumn to spring underwent gonadal recrudescence by spring. Although temperatures increased in nature during this time, Bullough concluded that, since the short photoperiod did not prevent recrudescence, this minnow has an internal reproductive rhythm which acts independently of exogenous factors. Kawamura and Otsuka (1950) demonstrated that long photoperiods and warm temperatures during winter and spring result in sexual maturation in female goldfish, *Carassius auratus.* No attempt was made to determine the relative importance of these two environmental factors, or if there is a seasonal variation in responsiveness to photoperiod and temperature. Fenwick (1970) exposed goldfish to various photoperiods at several different times of the year. Long photoperiods stimulated gonadal maturation in *Carassius*, but only during spring. All of Fenwick's experiments were conducted at low temperatures so the effects of warm temperatures in combination with different photoperiods are not known. The data of Kawamura and Otsuka (1950) indicate, in fact, that long photoperiods can stimulate gonadal maturation in *Carassius* during the winter if temperatures are warm.

The bridle shiner, *Notropis bifrenatus* (another cyprinid), exposed to constant warm temperatures in autumn exhibited sexual maturation ahead of the natural cycle if treated with a long photoperiod, but not on a short photoperiod (Harrington, 1950, 1957). Since low temperature controls were not included in these studies, no conclusions can be drawn as to whether the effect of long photoperiods is temperature dependent. Verghese (1967) reported that long photoperiod treatment in autumn brought *Cirrhina reba,* a subtropical cyprinid, into spawning condition. Verghese failed to mention the temperature conditions of the experiment, so the importance of temperature and the possible temperature dependency of photoperiodism is not known. Possible seasonal variation in gonadal responsiveness of *Cirrhina* to exogenous factors was not considered by Verghese.

In another cyprinid, the lake chub (*Couesius plumbeus*), low temperatures favor the meiotic phase (proliferation of spermatocytes) of spermatogenesis and high temperatures promote spermiation and proliferation of spermatogonia (Ahsan, 1966). Photoperiod alone does not stimulate any phase of spermatogenesis in *Couesius,* but at low temperatures short photoperiods have a slight acceleratory effect. In this species the meiotic and maturational stages of spermatogenesis can be exogenously stimulated only as the normal spawning season approaches. Although both photoperiod and temperature may be important reproductive regulatory factors in the cyprinid family, the lack of adequate controls in many experiments with minnows prohibit meaningful interpretation or generalizations. Furthermore, the effects of photoperiod and temperature on gonadal activity may vary with season. Unfortunately the seasonal aspect of exogenous regulation of teleost reproductive cycles has not been extensively examined.

The intent of the present investigation was to examine the effects of various photoperiod-temperature regimes on gonadal activity in the cyprinid, *Notemigonus crysoleucas,* during several different phases of the annual reproductive cycle.

Materials and Methods

Samples of *Notemigonus crysoleucas* were collected in ponds around the area of Menomonee Falls, Wisconsin (43°10′N) at several different times during the year and thus in different phases of the reproductive cycle. The reproductive cycle of this population of *Notemigonus* consists of a spawning season which extends from May through July. There is a postspawning season during August and September in which the gonads of this species are regressed. From October through February there is a gonadal preparatory period, in which spermatogonia proliferate slowly and spermatocytes appear in the testes. Vitellogenesis is initiated during this period. March and April can be referred to as the prespawning period; during this time final gonadal maturation occurs (*i.e.*, spermatozoa fill the testes and ovaries are distended with mature oocytes). Several fish from each nature sample were sacrificed and the gonads examined at the time of collection; these fish served as a reference for the experiments that followed. In the following discussion the fish sacrificed at the time of collection will be identified as initial controls.

Experimental fish were maintained under various photoperiod and constant temperature regimes (see results) in 114 or 285 liter tanks supplied with aerated and filtered dechlorinated tap water. Temperatures selected for these experiments are within the range normally experienced during the year in nature by this species. Illumination was a combination of incandescent and cool white fluorescent bulbs which gave a light intensity of 200 to 300 lux at the surface of each tank. Fish were fed once a day *ad libitum* on a commercial fish food (Tetra-Min). All specimens of *Notemigonus* used in these studies weighed between 12 and 17 grams.

The effects of the photoperiod-temperature regimes on reproductive function were assessed by gravimetric and histological techniques. Fish were sacrificed by severing the spinal cord. Body weight and gonadal weight were recorded immediately after sacrifice. Gravimetric data are expressed in terms of the gonosomatic index (gonadal weight/body weight × 100) since gonadal size in this species depends on body weight. After weighing, gonads were fixed in Bouin's solution and embedded in paraplast for histological examination. Spermatogenesis and oogenesis were each separated into seven and six recognizable phases respectively (Table I) to facilitate quantitative evaluation of gametogenic activity. Stage IV (active vitellogenic phase) of the oogenesis categories refers to ovaries in which yolky oocytes with a diameter of 125 to 620 μ predominate. Since Stage IV encompasses such a large size range of yolky oocytes a system has been adopted to better differentiate ovaries in this phase of maturation. The diameter of the 25 largest yolky oocytes in ovaries categorized in Stage IV were measured. A mean oocyte diameter was then determined for the ovaries of each fish in Stage IV.

TABLE I

Criteria used for evaluating gametogenic activity in the gonads of Notemigonus.

Stage	Histological characteristics of testes
0	"Regressing testis." Seminiferous lobules characterized by large numbers of pynotic nests of degenerating cells. Germinal epithelium disorganized.
1	"Quiescent testis." Germinal epithelium consists of a few primary spermatogonia only. Seminiferous lobules small in diameter. Lumen of the lobules contain only few residual spermatozoa.
2	"Mitotic phase." Similar to Stage 1 except spermatogonia are more numerous and mitotic figures are observed in many spermatogonia.
3	"Meiotic phase." Germinal epithelium consists of spermatogonia and spermatocytes. Testicular lobules larger than in Stages 1 and 2.
4	"Spermiogenic phase." Similar to Stage 3 except spermatids appear in germinal epithelium and some spermatozoa present in lumen of lobules.
5	"Prespawning testis." Seminiferous lobules very large and distended with sperm.
6	"Postspawning testis." Seminiferous lobules small and devoid of most germ cells. Sperm duct expanded and containing residual sperm.

Stage	Histological characteristics of ovaries
I	"Regressing ovary." Atretic follicles predominate in the ovary.
II	"Oogonial proliferation phase." Ovary characterized by nonyolky oocytes. Granulosa not fully organized around developing oocytes.
III	"Early vitellogenic phase." Oocytes with yolk vesicles present only in the periphery of ooplasm; diameter 70 to 125 μ.
IV	"Phase of active vitellogenesis." Yolk vesicles appear throughout ooplasm; diameter 125 to 620 μ.
V	"Prespawning ovary." Ovary charcterized by oocytes with a diameter of greater than 620 μ.
VI	"Postspawning ovary." The ovary appears red in color. The tunica albuginea thick, highly vascularized and folded. Post-ovulatory follicles predominate in the ovary. Ovarian stroma appears disorganized, yet highly vascularized.

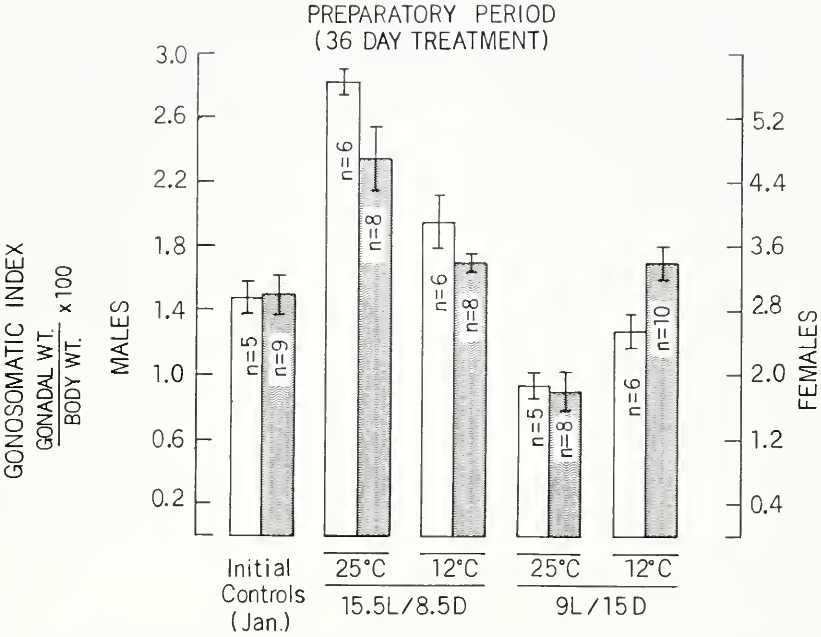


FIGURE 1. The effects of various photoperiod-temperature regimes on GSI in *Notemigonus* during the gonadal preparatory period. Initial controls refer to animals sacrificed at the time of collection. Histograms represent mean GSI; the mean is bracketed by one standard error. Testicular GSIs are shown by open histograms and ovarian GSIs by hatched histograms.

The mean yolky oocyte diameters from individual fish were averaged to obtain a mean and standard error for different experimental groups.

RESULTS

Preparatory season

The effects of various photoperiod-temperature regimes on gonadal activity in specimens of *Notemigonus* were first examined during January (late gonadal preparatory period). Fish were exposed for 36 days to a 15.5L/8.5D or 9L/15D photoperiod at either 25° or 12° C.

Testicular and ovarian GSIs increased significantly ($P < 0.01$) in fish maintained on the 15.5L/8.5D—25° C regime compared to the initial controls (Fig. 1). The testes of all initial controls were in the meiotic phase (Stage 3) of spermatogenesis (Table II). All male fish exposed to the long photoperiod-warm temperature regime advanced to the prespawning condition (Stage 5). Ovaries of the initial controls in January were in Stage III or IV (Table II). Three of the eight female fish maintained on the 15.5L/8.5D—25° C advanced to the prespawning condition (Stage V); the ovaries of the remaining fish in this group were in Stage IV (Table II). Stage IV oocytes in the ovaries of fish exposed to

TABLE II

Effects of various photoperiod and temperature regimes on gonadal activity in Notemigonus *during the preparatory period—36 day treatment (figures in this table refer to the number of fish in each group with gonads in a specific maturational stage).*

Treatment	Maturation stage of testes*						
	0	1	2	3	4	5	6
Initial controls (January)	0	0	0	5	0	0	0
15.5L/8.5D Photoperiod							
25° C	0	0	0	0	0	6	0
12° C	0	0	0	6	0	0	0
9L/15D Photoperiod							
25° C	3	0	2	0	0	0	0
12° C	2	0	4	0	0	0	0

	Maturation stage of ovaries*					
	I	II	III	IV	V	VI
Initial controls (January)	0	0	6	3(422 ± 16)[a]	0	0
15.5L/8.5D Photoperiod						
25° C	0	0	0	5(534 ± 23)[a]	3	0
12° C	5	0	2	3(500 ± 13)[a]	0	0
9L/15D Photoperiod						
25° C	5	3	0	0	0	0
12° C	0	0	4	6(431 ± 14)[a]	0	0

* See Table I.

[a] Mean diameter (±S.E.) in microns of 25 largest oocytes in each ovary.

the long photoperiod-warm temperature regime were significantly ($P < 0.05$) larger than in ovaries of the initial controls. These data indicate that a long photoperiod in combination with warm temperatures can promote final gonadal maturation in *Notemigonus* during the preparatory period.

Ovarian GSI in fish exposed to a long photoperiod at 12° C during the preparatory period did not differ significantly from the GSI of initial controls (Fig. 1). The ovaries of fish maintained on the 15.5L/8.5D—12° C regime were in Stages I, III or IV (Table II). Under these conditions testicular GSI increased significantly ($P < 0.05$) compared to the initial controls (Fig. 1). Testicular GSI in fish exposed to the 15.5L/8.5D photoperiod was significantly ($P < 0.05$) lower in the group at 12° C than in the group at 25° C. The testes of all fish exposed to the 15.5L/8.5D—12° C regime were in Stage 3 (Table II). Therefore, long photoperiod alone will not promote final gonadal maturation in *Notemigonus* during the preparatory period.

Ovarian and testicular GSIs decreased significantly ($P < 0.05$) in animals maintained on the 9L/15D—25° C regime compared to the initial controls (Fig. 1). Three of the male fish exposed to this regime were undergoing testicular regression (Stage 0) and the testes of the other two fish in this group were in Stage 2 (Table II). The ovaries of fish maintained on the 9L/15D—25° C regime were regressing (Stage I) or in Stage II (Table II). Thus, warm temperature alone will not promote final gonadal maturation in *Notemigonus* during the preparatory period.

Neither ovarian nor testicular GSI of fish exposed to the 9L/15D—12° C regime differed significantly from the GSIs of the initial January controls (Fig. 1). Histological examination of the gonads of the animals maintained on this regime showed that they were in essentially the same phases of maturation as the gonads of the initial controls (Table II).

Early prespawning season

Specimens of *Notemigonus* collected in early March were exposed for 21 days to the four photoperiod-temperature regimes mentioned above.

Ovarian and testicular GSIs of fish maintained on the 15.5L/8.5D—25° C regime were significantly ($P < 0.05$) lower than those of the initial controls (Fig. 2). The testes of all of the initial controls were in Stage 3 (Table III). Two male fish exposed to the long photoperiod-warm temperature regime spawned; the testes of other males in this group were in Stage 3, 4 or 5. Possibly the two fish of this experimental group in Stage 3 spawned and reinitiated spermatogenesis. Female fish in the initial March controls were all characterized by ovaries in the vitellogenic Stage IV (Table III). Four of the eight female fish maintained on the 15.5L/8.5D—25° C regime spawned. The relatively low gonadal weights in animals maintained on the long photoperiod-warm temperature regime were un-

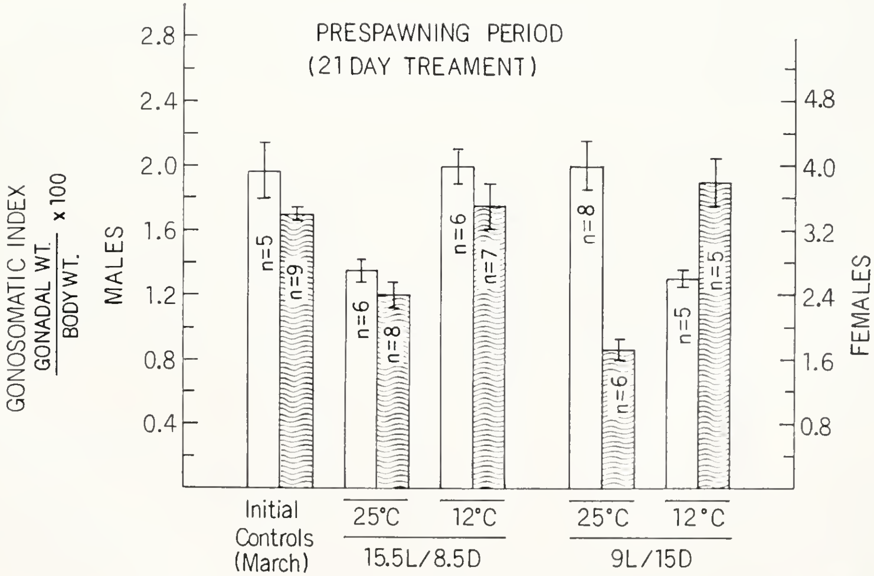


FIGURE 2. The effects of various photoperiod-temperature regimes on GSI in *Notemigonus* during the prespawning period. Initial controls refer to animals sacrificed at the time of collection. Histograms represent mean GSI; the mean is bracketed by one standard error. Testicular GSIs are shown by open histograms and ovarian GSIs by hatched histograms.

TABLE III

Effects of various photoperiod and temperature regimes on gonadal activity in Notemigonus *during the prespawning period—21 day treatment (figures in this table refer to the number of fish in each group with gonads in a specific maturational stage).*

Treatment	Maturation stage of testes*						
	0	1	2	3	4	5	6
Initial controls (March)	0	0	0	5	0	0	0
15.5L/8.5D Photoperiod							
25° C	0	0	0	2	1	1	2
12° C	4	0	0	2	0	0	0
9L/15D Photoperiod							
25° C	8	0	0	0	0	0	0
12° C	0	0	0	5	0	0	0

Treatment	Maturation stage of ovaries*					
	I	II	III	IV	V	VI
Initial controls (March)	0	0	0	9(388 ± 16)[a]	0	0
15.5L/8.5D Photoperiod						
25° C	0	0	0	2(531 ± 11)[a]	2	4
12° C	0	0	1	6(443 ± 27)[a]	0	0
9L/15D Photoperiod						
25° C	3	0	3	0	0	0
12° C	0	0	0	5(418 ± 24)[a]	0	0

* See Table I.

[a] Mean diameter (±S.E.) in microns of 25 largest oocytes in each ovary.

doubtedly due to spawning of some individuals in this group. These data further confirm that in combination with warm temperatures, long photoperiods stimulate final gonadal maturation and spawning in *Notemigonus.*

The 15.5L/8.5D—12° C regime did not cause significant changes in ovarian or testicular weights during the early prespawning period (Fig. 2). The testes of four of six fish maintained under these conditions regressed (Table III). Ovaries of a majority of the fish exposed to the 15.5L/8.5D—12° C regime were in Stage IV, as were those of the initial controls. Stage IV oocytes were, however, significantly ($P < 0.05$) larger in the experimental fish than in the initial controls (Table III).

Ovarian GSI was significantly ($P < 0.01$) lower in females maintained on the 9L/15D—25° regime than in initial controls (Fig. 2). Furthermore, the fish in this group were characterized by regressing ovaries (Stage I) or ovaries in Stage III (Table III). GSIs in male fish exposed to the short photoperiod-warm temperature regime and in initial controls were not significantly different (Fig. 2). Nonetheless, testicular regression (Stage 0) was occurring in all animals maintained on this regime (Table III).

GSIs of female fish acclimated to the 9L/15D—12° C regime and of initial controls did not differ significantly (Fig. 2). Moreover, ovarian activity in these two groups did not differ appreciably as determined by histological examination (Table III). In specimens of *Notemigonus* exposed to the short photoperiod-low temperature regime testicular GSI was significantly ($P < 0.01$) lower than in initial controls (Fig. 2). The testes of all fish in 9L/15D—12° C experimental group and in the initial control group were in Stage 3; the lower testicular weights in the experimental group indicate that spermatogenic activity is in part suppressed by these conditions.

Early spawning season

The effects of various photoperiod-temperature regimes on gonadal activity were again examined in *Notemigonus* collected in late April (early spawning season). Animals were exposed for 21 days to a 15.5L/8.5D or 9L/15D photoperiod at either 25° or 15° C.

Ovarian and testicular GSIs did not differ significantly in the group maintained on the 15.5L/8.5D—25° C regime and in the initial controls (Fig. 3). In the initial controls, the testes of four of six fish were in the prespawning condition, whereas all fish exposed to the long photoperiod-warm temperature regime were

TABLE IV

Effects of various photoperiod and temperature regimes on gonadal activity in Notemigonus *during the early spawning period—21 day treatment (figures in this table refer to the number of fish in each group with gonads in a specific maturational stage).*

Treatment	Maturation stage of testes*						
	0	1	2	3	4	5	6
Initial controls (Late April)	0	0	0	0	2	4	0
15.5L/8.5D Photoperiod							
25° C	0	0	0	0	0	7	0
15° C	0	0	0	0	3	2	0
9L/15D Photoperiod							
25° C	0	0	0	1	4	0	0
15° C	0	0	0	0	6	0	0

	Maturation stage of ovaries*					
	I	II	III	IV	V	VI
Initial controls (Late April)	0	0	0	2(496)[a]	4	0
15.5L/8.5D Photoperiod						
25° C	0	0	0	0	4	3
15° C	0	0	0	3(489 ± 29)[a]	3	0
9L/15D Photoperiod						
25° C	0	0	5	4(453 ± 17)[a]	0	0
15° C	0	0	3	2(508)[a]	0	0

* See Table I.

[a] Mean diameter (±S.E.) in microns of 25 largest oocytes in each ovary.

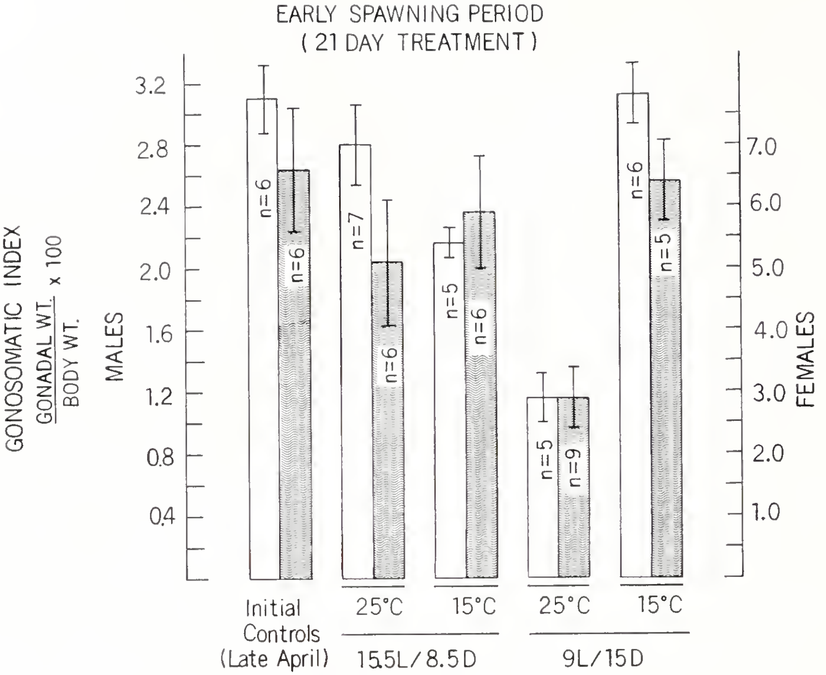


FIGURE 3. The effects of various photoperiod-temperature regimes on GSI in *Notemigonus* during the early spawning season. Initial controls refer to animals sacrificed at the time of collection. Histograms represent mean GSI; the mean is bracketed by one standard error. Testicular GSIs are shown by open histograms and ovarian GSIs by hatched histograms.

in the prespawning condition (Table IV). Four of the six female fish in the initial controls were also in the prespawning condition. Three of the seven female fish maintained on the 15.5L/8.5D—25° C regime spawned; the ovaries of the remaining animals in this group were in the prespawning condition (Table IV).

Testicular, but not ovarian GSI, was significantly ($P < 0.05$) lower in fish exposed to the 15.5L/8.5D—15° C regime than in initial controls (Fig. 3). Histological examination of the gonads of fish in the long photoperiod-low temperature group revealed that there were no striking differences in gametogenic activity compared to the initial controls (Table IV).

Both testicular and ovarian weights in the 9L/15D—25° C experimental group were significantly ($P < 0.01$) lower than in the initial controls (Fig. 3). The ovaries of five of the nine fish on the short photoperiod-warm temperature regime were in Stage III (Table IV). The testes of fish in this group were in Stage 3 or 4 (Table IV).

Neither ovarian nor testicular GSI in the animals maintained on the 9L/15D—15° C regime differed appreciably from GSIs of initial controls (Fig. 3). However, the ovaries of fish in the short photoperiod-low temperature group were in Stage III or IV (Table IV). The testes of all fish exposed to this regime were in Stage 4.

Postspawning season

Specimens of *Notemigonus* collected during mid-August (postspawning period) were exposed for 34 days to the photoperiod-temperature regimes mentioned above. Table V summarizes the changes in GSIs of fish maintained on these regimes.

Ovarian and testicular GSIs increased significantly ($P < 0.01$) in fish exposed to the 15.5L/8.5D—25° C regime compared to the initial controls (Table V). The ovaries of a majority of the initial controls were in a quiescent phase (Stage II), whereas those of females on the long photoperiod-warm temperature regime were in Stage IV or V (Table VI). Testes of initial controls were in Stage 1, 2 or 3; the 15.5L/8.5D—25° C regime stimulated the advancement of testicular development to Stage 4 (Table VI).

Testicular, but not ovarian GSI also increased significantly ($P < 0.01$) in animals exposed to the 15.5L/8.5D—15° C regime compared to the initial controls (Table V). Testes of all fish maintained on this regime were in Stage 4, whereas ovaries were in Stage III or IV (Table VI).

GSIs of both male and female *Notemigonus* exposed to a short photoperiod (at 25° and 15° C) were not significantly different from the GSIs of the initial controls. Histological examination of the gonads of fish exposed to a short photoperiod at either 25° or 15° C revealed that gametogenesis had not been stimulated under these conditions (Table VI).

DISCUSSION

The data presented here indicate that both photoperiod and temperature are important factors in regulating sexual cycling in the cyprinid teleost, *Notemigonus crysoleucas*. Furthermore, the effects of various photoperiod-temperature regimes on gonadal activity in *Notemigonus* appear to vary with season (*i.e.*, the gonadal condition of the initial controls). During the preparatory period, an "out-of-season" long photoperiod-warm temperature regime stimulates testicular and

TABLE V

Effects of various photoperiod and temperature regimes on GSI in Notemigonus *during the postspawning period—34 day treatment.*

Treatment	Gonosomatic index $\frac{\text{Gonadal weight}}{\text{Body weight}} \times 100$			
	n	Males ($\bar{X} \pm$ S.E.)	n	Females ($\bar{X} \pm$ S.E.)
Initial controls (mid August)	8	0.47 ± 0.06	10	1.12 ± 0.16
15.5L/8.5D Photoperiod				
25° C	7	3.00 ± 0.21*	8	4.51 ± 0.44*
15° C	5	2.12 ± 0.17*	5	2.33 ± 0.63
9L/15D Photoperiod				
25° C	5	0.42 ± 0.07	5	1.32 ± 0.19
15° C	4	0.57 ± 0.14	5	1.43 ± 0.22

* Significantly ($P < 0.01$) different than in initial controls.

TABLE VI

Effects of various photoperiod and temperature regimes on gonadal activity in Notemigonus *during the postspawning period—34 day treatment (figures in this table refer to the number of fish in each group with gonads in a specific maturational stage).*

Treatment	Maturation stage of testes*						
	0	1	2	3	4	5	6
Initial controls (mid August)	0	2	4	2	0	0	0
15.5L/8.5D Photoperiod							
25° C	0	0	0	0	7	0	0
15° C	0	0	0	0	5	0	0
9L/15D Photoperiod							
25° C	0	2	2	1	0	0	0
15° C	0	0	2	2	0	0	0

	Maturation stage of ovaries*					
	I	II	III	IV	V	VI
Initial controls (mid August)	0	6	3	1(363)[a]	0	0
15.5L/8.5D Photoperiod						
25° C	0	0	1	4(396 ± 21)[a]	3	0
15° C	0	0	2	3(367 ± 14)[a]	0	0
9L/15D Photoperiod						
25° C	0	1	4	0	0	0
15° C	0	3	2	0	0	0

* See Table I.

[a] Mean diameter (±S.E.) in microns of 25 largest oocytes in each ovary.

ovarian development to the prespawning condition. Therefore, a long photoperiod in combination with warm temperatures is apparently required for final gonadal maturation in this species. Neither a long photoperiod alone nor a warm temperature alone induce gonadal development to the prespawning condition during the preparatory period. A long photoperiod-warm temperature regime also promotes sexual maturation in two other cyprinid fish, *Carassius auratus* (Kawamura and Otsuka, 1950) and *Notropis bifrenatus* (Harrington, 1950, 1957) during winter and spring. A combination of long photoperiods and warm temperatures are also required for final gonadal maturation in the centrarchid, *Lepomis cyanellus* (Kaya and Hasler, 1972). Other investigators (Bullough, 1939; Verghese, 1967) have suggested that long photoperiod treatment can stimulate "out-of-season" final gonadal maturation in cyprinids; these investigators, however, failed to mention the temperatures at which their experiments were conducted, so the relative importance of photoperiod and temperature cannot be determined.

During the prespawning and spawning seasons a warm temperature-long photoperiod regime stimulates spawning in *Notemigonus*. Long photoperiods alone or warm temperatures alone do not induce spawning during these periods. Apparently then, spawning in *Notemigonus* depends primarily on photoperiod and temperature conditions. In addition, these data suggest that a combination of a long photoperiod and warm temperature are required for spawning. In the postspawning season a long photoperiod-warm temperature regime can induce spermiogenesis and stimulate development of ovaries to the prespawning condition. These data indicate that *Notemigonus* is not totally refractory to these conditions. In nature gonadal regression in *Notemigonus* occurs in late July and September when environmental temperatures are warm, but when daylength begins to decrease. Potentially then, *Notemigonus* could continue to breed if daylength did not decrease.

Short photoperiods or decreasing daylength in combination with warm temperatures may induce gonadal involution in *Notemigonus*. In fact, short photoperiod-warm temperature treatment did result in gonadal regression in *Notemigonus* during the preparatory and prespawning periods. The gonadal regression response to a warm temperature-short photoperiod regime was not, however, observed during the spawning and postspawning periods. During these periods exposing *Notemigonus* to a short photoperiod-warm temperature regime did not stimulate advancement to the prespawning condition, but did promote spermatocyte proliferation and the initiation of vitellogenesis. These observations are consistent with environmental data because spermatocyte proliferation and the initiation of vitellogenesis do occur in early fall. It appears, therefore, that the early phases of gametogenesis are independent of environmental factors (more evidence for this hypothesis is presented below). The potential role of environmental factors in controlling gonadal regression in teleosts has received very little attention. In *Gillichthys mirabilis* (de Vlaming, 1972b) and *Lepomis cyanellus* (Kaya, 1973) high temperatures induce gonadal regression.

Long photoperiods in the absence of warm temperature do not promote final gonadal maturation in *Notemigonus*. A long photoperiod in combination with a low temperature maintains vitellogenesis during all seasons in *Notemigonus*, but does not promote final ovarian maturation or spawning. Therefore, long photoperiods alone cannot induce final oocyte maturation in this species. During the preparatory and prespawning seasons a long photoperiod-low temperature regime is not effective in stimulating spermiogenesis in *Notemigonus*. During the spawning and postspawning periods this regime maintains gametogenesis, but does not promote final testicular maturation. These data indicate that the effects of a long photoperiod-low temperature regime on gonadal development in *Notemigonus* vary with season, yet these conditions will never stimulate final gonadal maturation or spawning.

A short photoperiod-low temperature regime is effective in maintaining vitellogenesis at all times of the year in *Notemigonus*. Final ovarian maturation, however, will not occur in fish maintained under these conditions. During the preparatory, prespawning and postspawning seasons a low temperature-short photoperiod regime promotes spermatogonial and spermatocyte proliferation, but not spermiogenesis. Thus these conditions do not retard the early phases of gametogenesis in this cyprinid.

Combined, the results of these experiments indicate that spermiogenesis and vitellogenesis are independent of environmental control in *Notemigonus*, but spermiation and final oocyte maturation depend on a combination of long daylengths and warm temperatures.

The effects of various photoperiod and temperature regimes on gonadal activity in *Notemigonus* probably result from changes in pituitary gonadotropin secretion. Indeed, pituitary gonadotropin potency did vary in animals maintained under different regimes (de Vlaming, unpublished results). In *Notemigonus* hypothalamic gonadotropin releasing activity is significantly greater in fish exposed to a warm temperature-long photoperiod regime than in animals maintained on a warm temperature-short photoperiod regime (de Vlaming, unpublished results). The long photoperiod condition is also effective in stimulating spawning, whereas the short photoperiod regime results in gonadal regression. The effects of daylength on gonadal activity in *Notemigonus* thus seem to be mediated via the hypothalamus.

J. Flanagan, M. J. Vodicnik, R. J. Pardo, and G. Paquette offered valuable technical assistance. This work was supported by NSF Grant GB-41338.

SUMMARY

1. The effects of various photoperiod-temperature regimes on gonadal activity in the cyprinid teleost, *Notemigonus crysoleucas*, were examined during several different phases of the annual reproductive cycle.

2. Regardless of the time of year when the experiment is initiated a long photoperiod-warm temperature regime stimulates gonadal development to the prespawning condition or induces spawning. Neither a warm temperature alone nor a long photoperiod alone will stimulate final gonadal maturation.

3. *Notemigonus* is not "refractory" to long photoperiod-warm temperature gonadal activation during the postspawning season.

4. Short photoperiods in combination with warm temperatures cause gonadal regression in this species. A low temperature-short photoperiod regime does not induce gonadal involution.

5. Spermatocyte formation and proliferation as well as the early phases of vitellogenesis occur independently of environmental factors. Final gonadal maturation and the rate of gametogenesis, however, depend on specific environmental conditions.

LITERATURE CITED

AHSAN, S. N., 1966. Effects of temperature and light on the cyclical changes in spermatogenic activity of the lake chub, *Couesius plumbus*. *Can. J. Zool.*, **44**: 161–171.

BULLOUGH, W. S., 1939. A study of the reproductive cycle of the minnow in relation to the environment. *Proc. Zool. Soc. London*, **109A**: 79–102.

BULLOUGH, W. S., 1940. The effect of the reduction of light in spring on the breeding season of the minnow (*Phoxinus laevis*). *Proc. Zool. Soc. London*, **110A**: 149–157.

CHAMPY, M. C., 1923. Observations sur les caractères sexueles chez les poissions. *C. R. Seanc. Soc. Biol.*, **88**: 414–417.

DE VLAMING, V. L., 1972a. Environmental control of teleost reproductive cycles: a brief review. *J. Fish. Biol.*, **4**: 131–140.

DE VLAMING, V. L., 1972b. The effects of temperature and photoperiod on reproductive cycling in the estuarine gobiid fish, *Gillichthys mirabilis*. *Fish. Bull.* **70**: 1137–1152.

DE VLAMING, V. L., 1974. Environmental and endocrine control of teleost reproduction. Pages 13–83 in C. B. Schreck, Ed., *Control of sex in fishes*. Sea Grant and V.P.I. & S.U. Press. V.P.I.-S.G.-74-01.

Fenwick, J. C., 1970. The pineal organ: Photoperiod and reproductive cycles in the goldfish. *J. Endocrinol.,* **46**: 101–111.

Harrington, R. W., Jr., 1950. Preseasonal breeding by the bridled shiner, *Notropis bifrenatus* induced under light-temperature control. *Copeia,* **1950**: 304–311.

Harrington, R. W., Jr., 1957. Sexual photoperiodicity of the cyprinid fish, *Notropis bifrenatus* in relation to the phase of its annual reproductive cycle. *J. Exp. Zool.,* **135**: 529–553.

Kawamura, T., and S. Otsuka, 1950. On acceleration of the ovulation in the goldfish. *Jap. J. Ichthyol.,* **1**: 157–165.

Kaya, C. M., 1973. Effects of temperature and photoperiod on seasonal regression of gonads of green sunfish, *Lepomis cyanellus*. *Copeia,* **1973**: 369–373.

Kaya, C. M., and A. D. Hasler, 1972. Photoperiod and temperature effects on the gonads of green sunfish, *Lepomis cyanellus* (Rafinesque), during the quiescent, winter phase of its annual cycle. *Trans. Am. Fish. Soc.,* **101**: 220–275.

Verghese, P. U., 1967. Prolongation of spawning season in the carp *Cirrhina reba*. (Ham.) by artificial light treatment. *Curr. Sci.,* **36**: 465–467.

MARINE BIOLOGICAL LABORATORY
LIBRARY
WOODS HOLE

Reference: *Biol. Bull.*, **148**: 416–428. (June, 1975)

METABOLIC SIGNIFICANCE IN NUCLEIC ACID METABOLISM AND PROTEIN SYNTHESIS OF DIETARY AMP REQUIREMENT IN *ARTEMIA SALINA* (L.)

A. HERNANDORENA

Laboratoire du Museum National d'Histoire Naturelle au Centre d'Etudes et de Recherches Scientifiques de Biarritz, 64, France

The absolute and specific requirement of *Artemia* for a dietary purine nucleotide has been demonstrated. AMP deficiency reduces growth rate and survival percentage, induces a supernumerary gonopode morphogenesis and reduces abdominal length. The purine requirement can be satisfied by IMP, AMP or GMP; adenine, guanine and adenosine are metabolized but fulfill the requirement less effectively (Hernandorena, 1972b). The need for an energetic nutrient plus AMP/albumin ratio has been shown. With increasing salinity of the medium, growth rate is reduced, the abdomen grows relatively longer and the energetic nutrient plus AMP/albumin ratio is altered. The quantitative nature of AMP deficiency inducing the morphogenetic action depends on albumin concentration and salinity. Since oxidative metabolism and ATP production are required for amino acid incorporation into tissue proteins we suggested that AMP morphogenetic action should be looked for at the protein synthesis level (Hernandorena, 1974a).

The use of specific inhibitors seems to offer a specially useful approach to study the possible biochemical alterations underlying the AMP morphogenetic action. We have already effectively employed aminopterin and 5-fluorodeoxyuridine (5-FUDR) to elucidate the significance of *Artemia* folic acid requirement (Hernandorena, 1970, 1972a).

The essentiality of axenic conditions in these studies has to be underlined. The lack of activity of a number of analogues on *Oniscus asellus* growth suggests the existence of a high level of metabolite synthesis in the gut of this crustacean (Beerstecher, Cornyn and Vokmann, 1954). Fautrez-Firlefyn and Fautrez (1970) reported the remarkable lack of toxicity of hydroxyurea in *Artemia,* since viability is not affected by 48-hour immersion in M/100 and M/50 solutions.

In *Artemia,* feeding levels show a profound effect on growth both as it influences growth rate and final size attained (Mason, 1963). According to Dagg (1969), "*Artemia* protein concentration is highest during exponential growth indicating that almost all growth is due to new protein synthesis . . . the growth rates associated to a given RNA concentration range tremendously. The poor relationship between RNA/protein concentration and growth measured by protein increase is unexplainable. . . . The RNA-growth relationship is probably most valid under steady growth conditions". These are not the conditions used by the author.

In this paper we report the effects of various inhibitors affecting nucleic acid metabolism (Fig. 1) and protein synthesis. Our next paper will be concerned with the metabolic significance of dietary AMP in energy production in relation to temperature and salinity.

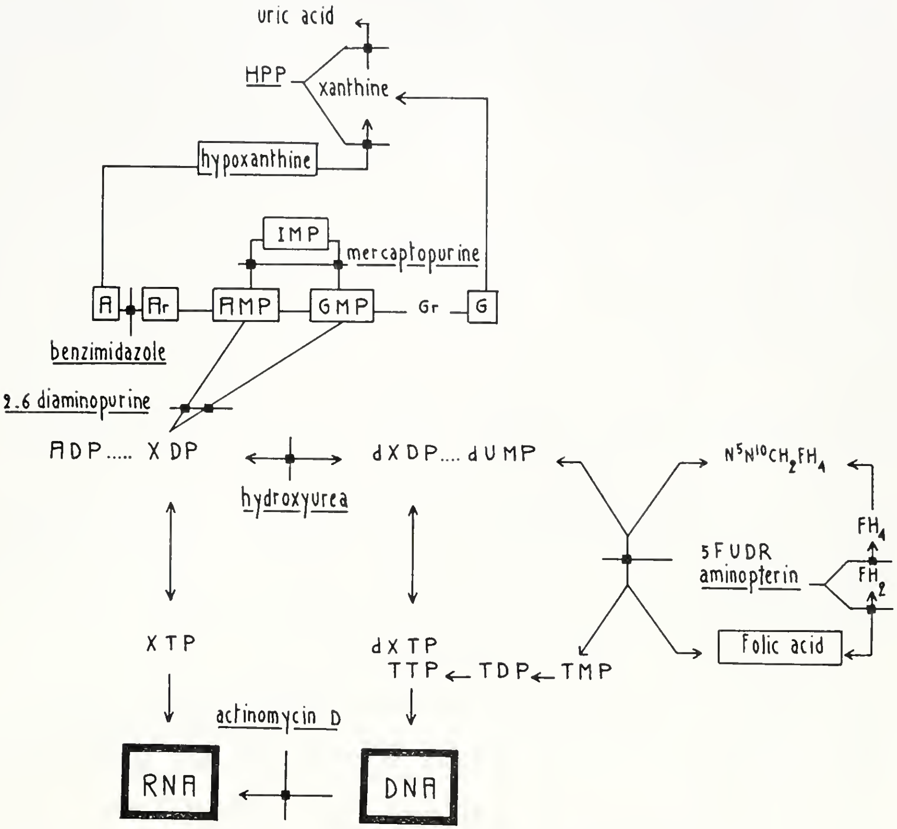

uric acid
HPP
xanthine
hypoxanthine
IMP
mercaptopurine
A
Ar
AMP
GMP
Gr
G
benzimidazole
2.6 diaminopurine
ADP..... XDP
hydroxyurea
dXDP.... dUMP
N5N10CH2FH4
FH4
5FUDR
aminopterin
FH2
Folic acid
XTP
dXTP
TTP
TDP
TMP
actinomycin D
RNA
DNA

FIGURE 1. Metabolic reactions of nucleic acid, as blocked by the inhibitors administrated, are shown; metabolites administered are indicated by boxed compounds; inhibitors are underlined; and points of inhibition are indicated by a solid square on the crossing lines.

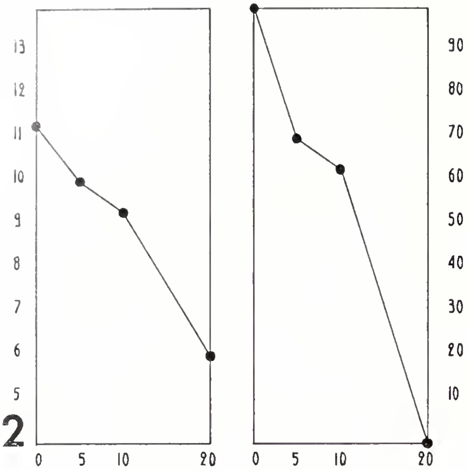
MATERIALS AND METHODS

The method developed by Provasoli and d'Agostino (1969) for the Utah strain axenic cultivation has been used and described previously (Hernandorena, 1970, 1972a, b, c). The inhibitors used are from Nutritional Biochemical Co.; HPP was graciously supplied by Burroughs Wellcome and Co.

RESULTS

Nucleic acid metabolism

Purine catabolism leads to xanthine dehydrogenase substrates hypoxanthine and xanthine. This enzyme is specifically inhibited by 4-hydroxy-pyrazolo-(3-4 d)-pyrimidine (HPP) and this inhibitor action has already been studied in *Artemia* (Hernandorena, 1972c). We intend to study HPP action on larvae for which purine requirement has been fulfilled by different purine derivatives.



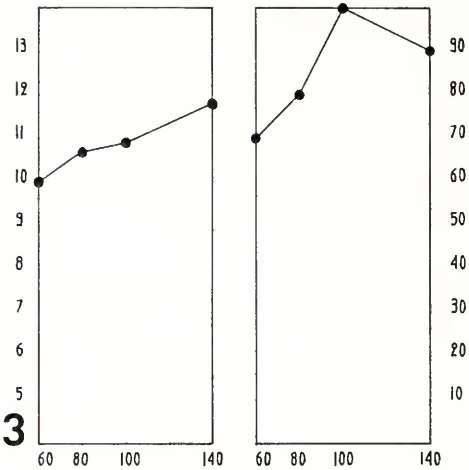
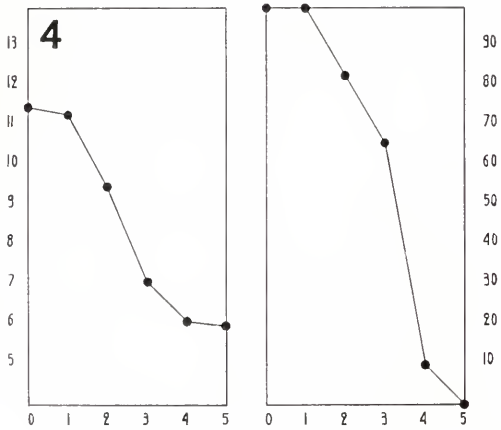


FIGURE 2. Effect of benzimidazole; both abscissae represent benzimidazole in mg%. In all figures, growth is represented in the left-hand, and survival in the right-hand graph, the left-hand ordinate being the growth index for the 14th day of development, and the right-hand ordinate being the survival percentage for index 10 (end of the larval life), see Hernandorena, 1972a.

FIGURE 3. Benzimidazole-AMP antagonism; both abscissae AMP in mg%; benzimidazole constant at 5 mg%; ordinates growth and survival as in Figure 2.

Benzimidazole reduces growth index and survival percentage (Fig. 2) but does not induce a supernumerary gonopode morphogenesis. The detrimental effect of benzimidazole is relieved by AMP (Fig. 3).



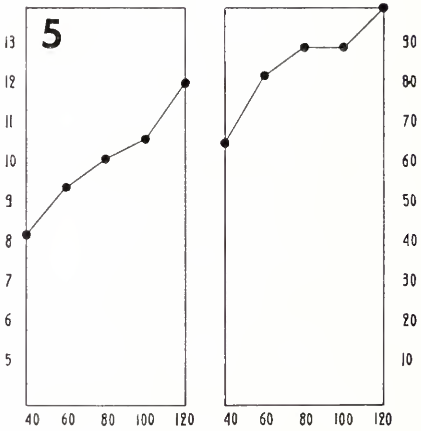
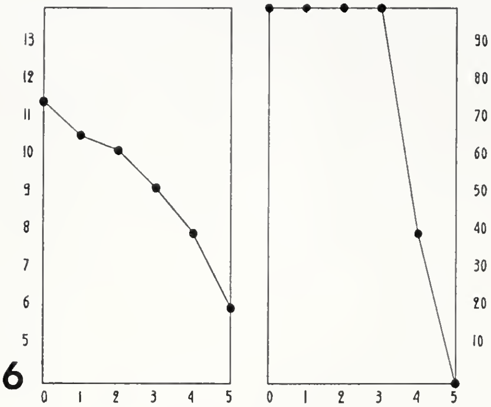


FIGURE 4. Effect of 2–6 diaminopurine; both abscissae 2–6 diaminopurine in mg%; ordinates growth and survival as in Figure 2.

FIGURE 5. 2–6 diaminopurine-AMP antagonism; both abscissae AMP in mg%; 2–6 diaminopurine constant at 2 mg%; ordinates growth and survival as in Figure 2.



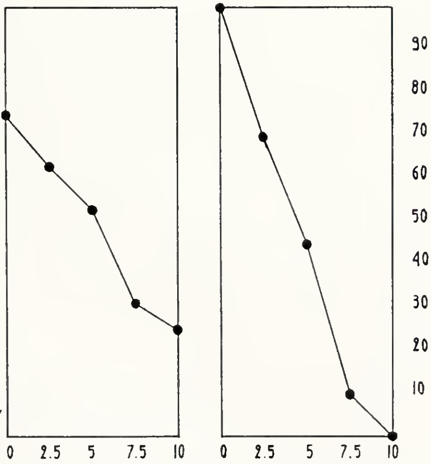
6

FIGURE 6. Effect of mercaptopurine; both abscissae mercaptopurine in mg%; no AMP present, IMP at 60 mg%; ordinates growth and survival as in Figure 2.

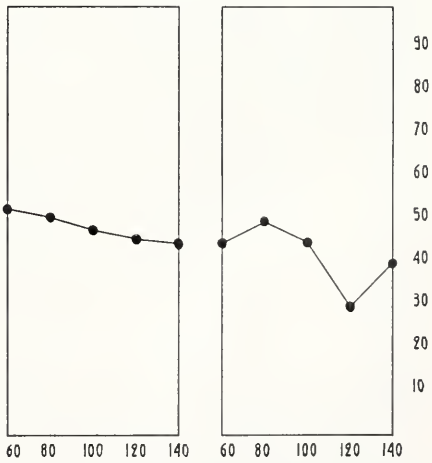
The inhibitory effect of 2–6 diaminopurine administration (Fig. 4) is relieved by AMP (Fig. 5). Morphogenesis is normal.

The 6-mercaptopurine action has been studied on larvae reared in a medium containing 60 mg% IMP instead of AMP. Figure 6 shows the detrimental effect of mercaptopurine: it reduces growth index and survival percentage but does not induce a supernumerary gonopode morphogenesis.

Figure 7 shows the inhibitory effect of hydroxyurea administration; this effect is not relieved by AMP (Fig. 8).



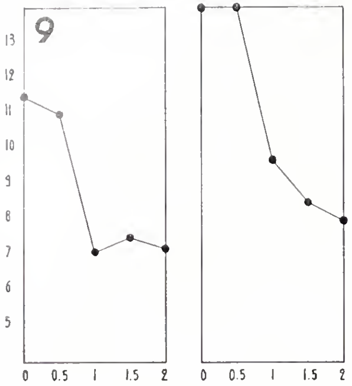
7



8

FIGURE 7. Effect of hydroxyurea; both abscissae hydroxyurea in mg%; ordinates growth and survival as in Figure 2.

FIGURE 8. Hydroxyurea-AMP antagonism; both abscissae AMP in mg%; hydroxyurea constant at 5 mg%; ordinates growth and survival as in Figure 2.



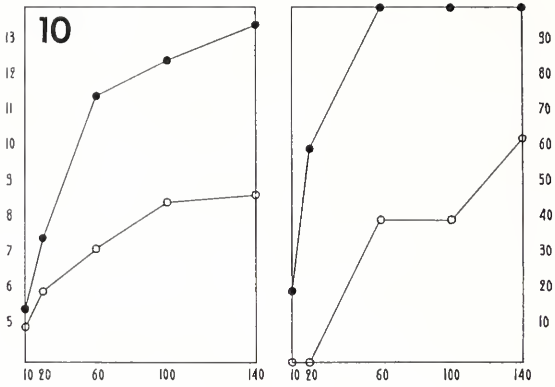
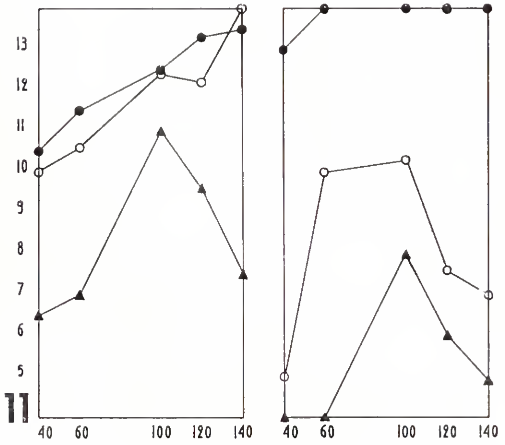


FIGURE 9. Effect of actinomycin D; both abscissae actinomycin in $\mu g\%$; ordinates growth and survival as in Figure 2.

FIGURE 10. Effect of AMP concentration; both abscissae AMP in mg%; closed circles, no addition; open circles, actinomycin D 2 $\mu g\%$; ordinates growth and survival as in Figure 2.

Actinomycin D reduces growth index and survival percentage (Fig. 9) and this action is dependent on AMP concentration (Fig. 10). This antibiotic does not induce a supernumerary gonopode morphogenesis. The result has been checked by rearing larvae in an actinomycin D-containing medium during different periods of the larval life. Morphogenesis is normal whatever the time spent in the presence of the antibiotic.

HPP action depends on AMP concentration (Fig. 11) and on adenine concentration (Fig. 12). Minimal action occurs at 100 mg% AMP and at a 10



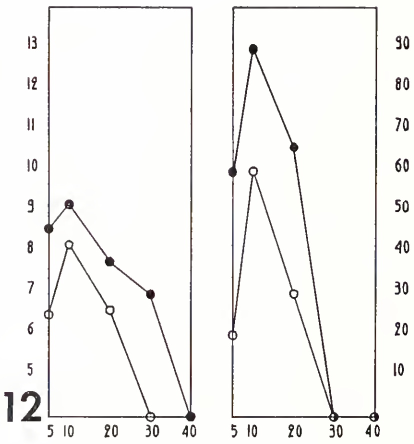
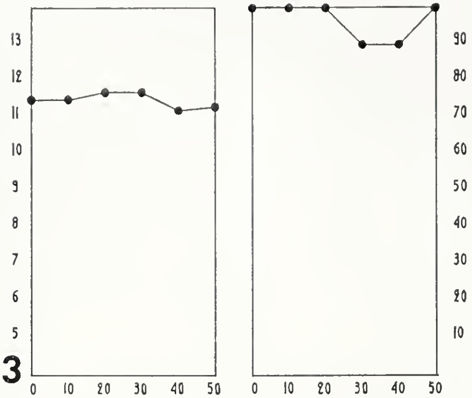


FIGURE 11. Effect of AMP concentration; both abscissae AMP in mg%; closed circles, no addition; open circles, HPP at 40 mg%; closed triangles, HPP at 80 mg%; ordinates growth and survival as in Figure 2.

FIGURE 12. Effect of adenine; both abscissae adenine in mg%; closed circles, no addition; open circles, HPP at 20 mg%; ordinates growth and survival as in Figure 2.



13

Figure 13. Effect of hypoxanthine; both abscissae hypoxantine in mg%; ordinates growth and survival as in Figure 2.

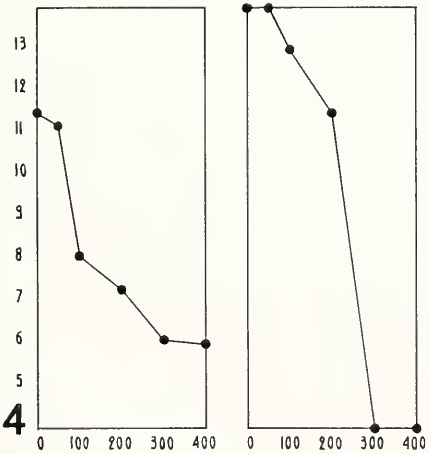
mg% adenine concentration. With increasing AMP or adenine concentration HPP action increases.

Hypoxanthine administration up to a 50 mg% level has no effect on growth rate and survival percentage (Fig. 13).

Protein synthesis

Protein synthesis has been studied by the use of the antibiotic puromycin and the amino acid analogue D.L. parafluorophenylalanine under different experimental conditions.

14



15

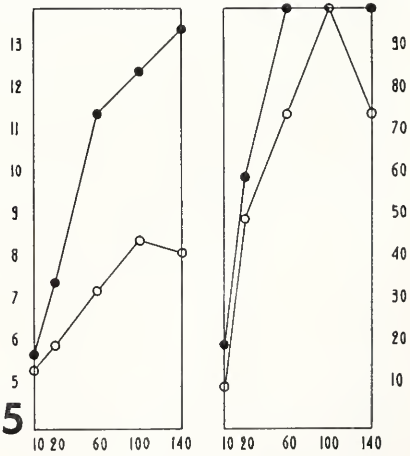
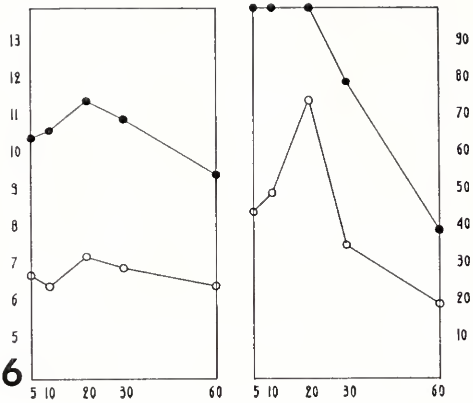


Figure 14. Effect of puromycin; both abscissae puromycin in μg%; ordinates growth and survival as in Figure 2.

Figure 15. Effect of AMP concentration; both abscissae AMP in mg%; albumin constant at 20 mg%; closed circles, no addition; open circles, puromycin at 200 μg%; ordinates growth and survival as in Figure 2.



16

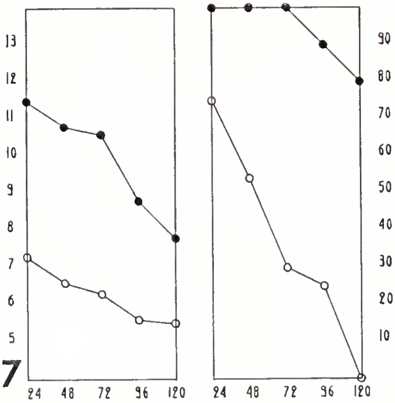
FIGURE 16. Effect of albumin concentration; both abscissae albumin in mg%; AMP constant at 60 mg%; closed circles, no addition; open circles, puromycin at 200 μg%; ordinates growth and survival as in Figure 2.

Puromycin administration reduces growth index and survival percentage (Fig. 14).

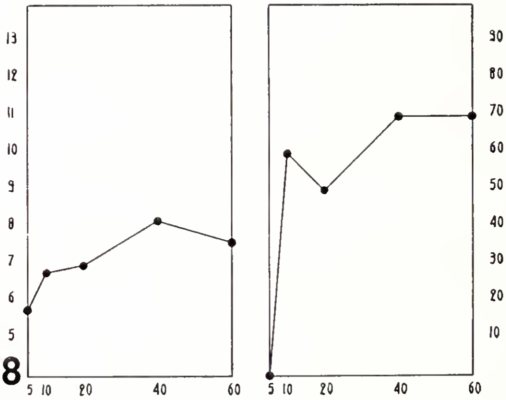
With albumin constant at 20 mg%, puromycin action decreases with increasing AMP concentration up to a 100 mg% level (Fig. 15).

With AMP constant at 60 mg%, puromycin action depends on albumin concentration, the minimal effect corresponding to a 20 mg% level (Fig. 16).

With increasing salinity, albumin constant at 20 mg% and AMP constant at 60 mg%, puromycin detrimental effect increases (Fig. 17).



17



18

FIGURE 17. Effect of salinity; both abscissae NaCl in g‰; albumin constant at 20 mg%; AMP constant at 60 mg%; closed circles, no addition; open circles, puromycin at 200 μg%; ordinates growth and survival as in Figure 2.

FIGURE 18. Effect of albumin concentration; both abscissae albumin in mg%, salinity at 120‰; closed circles, puromycin at 50 μg%; ordinates growth and survival as in Figure 2.

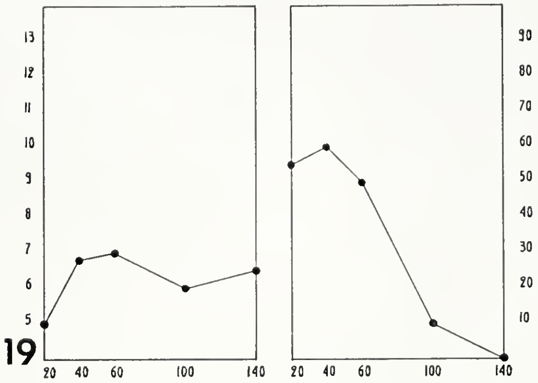


FIGURE 19. Effect of AMP concentration; both abscissae AMP in mg%; salinity at 120‰; closed circles, puromycin at 50 µg%; ordinates growth and survival as in Figure 2.

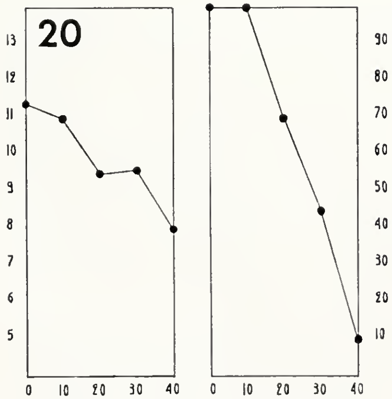
We have demonstrated that with increasing salinity, the albumin requirement increases and that the growth index increases with AMP concentration, provided enough albumin is supplied (Hernandorena, 1974a).

With salinity constant at 120‰, puromycin action depends on albumin concentration (Fig. 18) and AMP concentration (Fig. 19), minimal effect corresponding to a 40 mg% albumin level and a 40 mg% AMP concentration.

These results are confirmed by D.L. parafluorophenylalanine administration.

This analogue reduces growth index and survival percentage (Fig. 20).

At 24‰ salinity, minimal action corresponds to a 100 mg% AMP concentration (Fig. 21) and to a 20 mg% albumin concentration (Fig. 22).



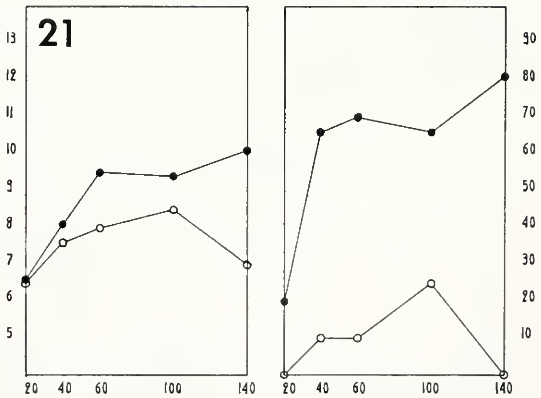
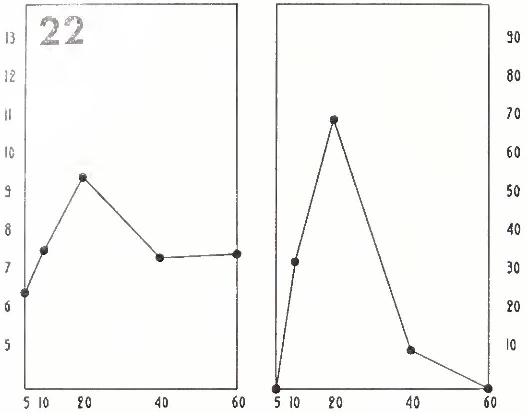


FIGURE 20. Effect of D.L. parafluorophenylalanine; both abscissae parafluorophenylalanine in mg%; ordinates growth and survival as in Figure 2.

FIGURE 21. Effect of AMP concentration; both abscissae AMP in mg%; albumin constant at 20 mg%; closed circles, parafluorophenyalanine 20 mg%; open circles, parafluorophenylalanine 40 mg%; ordinates growth and survival as in Figure 2.



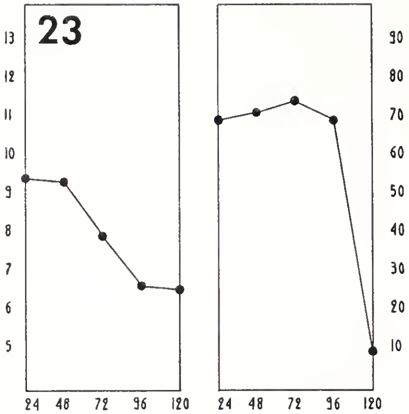
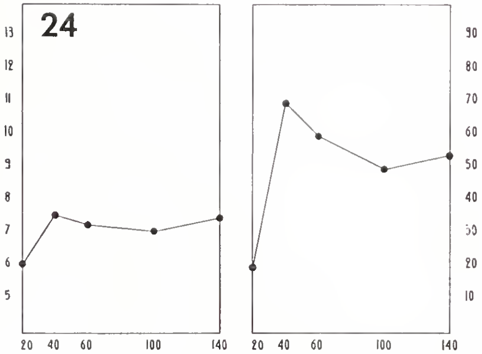


FIGURE 22. Effect of albumin concentration; both abscissae albumin in mg%; AMP constant at 60 mg%; closed circles parafluorophenylalanine 20 mg%; ordinates growth and survival as in Figure 2.

FIGURE 23. Effect of salinity; both abscissae NaCl in g‰; albumin constant at 20 mg%, AMP constant at 60 mg%; closed circles, parafluorophenylalanine 20 mg‰; ordinates growth and survival as in Figure 2.

With increasing salinity, parafluorophenylalanine detrimental effect increases (Fig. 23). At 120‰ salinity, minimal action corresponds to a 40 mg% AMP concentration (Fig. 24), and to a 40 mg% albumin concentration (Fig. 25), thus confirming the reduction in AMP requirement and increase in albumin requirement with increasing salinity.



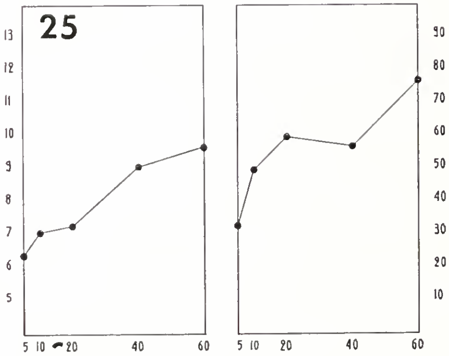


FIGURE 24. Effect of AMP concentration; both abscissae AMP in mg%; salinity at 120‰; closed circles, parafluorophenylalanine at 5 mg%; ordinates growth and survival as in Figure 2.

FIGURE 25. Effect of albumin concentration; both abscissae albumin in mg%; salinity at 120‰; closed circles, parafluorophenylalanine at 5 mg%; ordinates growth and survival as in Figure 2.

DISCUSSION

Since the specificity and essentiality of a purine nucleotide requirement has already been demonstrated by omission experiments (Hernandorena, 1972b), the growth inhibition observed after administration of the different inhibitors, does not bring any additional data. No morphogenetic effect is induced whatever the reaction blocked, and this negative result underlines the limits of the antagonists method.

The morphogenetic effects observed after DNA synthesis inhibition resulting from aminopterin or 5-FUDR administration have been described (Hernandorena, 1970, 1972a). We observed a teratogenic action with numerous appendicular malformations which are different from the supernumerary gonopodes induced by AMP deficiency. We can conclude that the morphogenetic action caused by AMP deficiency does not result from inhibition of DNA synthesis. This is confirmed by hydroxyurea administration, since hydroxyurea induced growth inhibition is not relieved by AMP.

Actinomycin D action depends on AMP concentration and this antibiotic does not induce any morphogenetic action.

We have demonstrated that a critical period exists for the induction of supernumerary gonopods. Animals reared on an AMP-deficient medium until growth index 7 and transferred at this stage to the basal medium show supernumerary gonopods like animals reared on an AMP-deficient medium until maturity (Hernandorena, 1974b). Actinomycin D administration restricted to the critical period does not induce supernumerary gonopods. This negative result underlines the problem of interpreting antibiotics action.

It is not clear how nucleotides would be superior to the bases or nucleosides since the former group of compounds are not effectively transported across cell membranes and probably must be catabolized to the nucleosides or bases prior to entering the cell (Kelley, 1972). We suggested that dietary bases are catabolized too rapidly (Hernandorena, 1972b). HPP action or xanthine dehydrogenase activity is dependent on the qualitative and quantitative nature of dietary purine derivatives. Hypoxanthine has no effect and this metabolite would not be metabolized (Stirpe and Della Corte, 1965). When adenine is supplied to meet the purine requirement, HPP action increases. This result could be interpreted as suggested by a rapid catabolism of dietary bases.

It is interesting to note that in different insects, xanthine dehydrogenase activity has been shown to vary with diet, especially with the protein composition of the diet (Ito and Mukaiyama, 1964; Villela, Calcagnotto, Piedras Lopes, and Rios Magalhaes, 1970) and that the dietary RNA requirement depends on the protein concentration of the diet (Geer, 1963).

In *Artemia* the quantitative nature of AMP requirement depends on the albumin concentration of the diet (Hernandorena, 1974a).

With albumin constant at 20 mg%, and provided enough energetic nutrients are supplied, growth rate increases with the AMP concentration up to a 140 mg% level and decreases thereafter. The major increase takes place between 20 and 60 mg% AMP (Hernandorena, 1974a). The abdomen grows relatively longer with increasing AMP concentration up to a 200 mg% level. With AMP concen-

TABLE I

Growth rate and abdominal length as percentage of total length (1 % L) in relation to AMP concentration and salinity.

Albumin constant at 20 mg% Starch constant at 100 mg%	Salinity						
	24‰						120‰
	AMP mg%						AMP mg%
	20	60	100	140	180	200	60
Growth index 14th day	7.5	11.5	12.5	13.5	12.3	11.7	7.8
1 % L	39.7	45.2	47.6	48	50	54	49.3

tration ranging from 140 mg% to 200 mg% growth rate decreases but abdominal length increases.

With AMP constant at 60 mg% and albumin constant at 20 mg% growth rate decreases and abdominal length increases with increasing salinity. These results are summarized in Table I. Part of them will be published separately. The abdominal length as percentage of total length (1% L) is reported and measured using the methods described by Gilchrist (1956).

These results show that AMP deficiency reduces growth index and abdominal length while salinity reduces growth index and increases abdominal length. So it seems that growth rate and abdominal length depend on two different metabolic systems.

At 24‰ salinity, maximal protein synthesis estimated by minimal puromycin and parafluorophenylalanine action is achieved with an AMP:albumin ratio standing somewhere at 100:20. This ratio does not correspond to maximal growth rate which is achieved with a 140:20 ratio, nor to maximal abdominal length which is achieved with a 200:20 ratio.

With increasing salinity, puromycin and parafluorophenylalanine detrimental effect increases. This result suggests that protein synthesis decreases with increasing salinity. We have demonstrated that protein synthesis depends on albumin concentration but albumin requirement increases with increasing salinity. With albumin constant at 20 mg% protein synthesis would decrease with increasing salinity.

Survival studies of newly hatched nauplii, incubated during 24 hours at various salinities in the presence of puromycin, chloramphenicol and cycloheximide, demonstrated that protein synthesis inhibitors are more effective at high salt concentration. The results are not due to changes in permeability to the inhibitors with varying salinity since there is no significant difference in the uptake of ^{3}H-puromycin by nauplii incubated at different salinities (Ewing, Peterson and Conte, 1972). In our experimental conditions, animals are feeding and the results obtained could be due to a different drinking rate resulting from the mechanisms of osmotic regulation (Hernandorena, 1974a) although Croghan (1958) stated that swallowing is continuous in all active animals whatever the salinity of the medium.

With salinity constant at 120‰, that is to say with a constant drinking rate, maximal protein synthesis is achieved with an AMP:albumin ratio standing somewhere at 40:20. So it can be concluded that for a given albumin concentration (20 mg%), salinity reduces the AMP requirement for maximal protein synthesis.

In *Bombyx mori*, larval growth depends on at least two metabolic systems; one associated with the length of early developmental period which is specially sensitive to carbohydrate metabolism, and the other responsible for an extra larval molting which is probably related to protein metabolism (Kato and Sumimoto, 1968). With increasing salinity, *Artemia* starch requirement decreases (Hernandorena, 1974a).

Before drawing any interpretation, conversion of these informations to energy flow must be introduced. The ratio between AMP concentration and ATP production should be considered. So information must be gained regarding the nature of substrates oxidized.

As pointed by House (1966), Sang (1959) stated that balance between nutrients deserves much more investigation because here one delves most closely into examination of metabolic processes. The influence of metabolic adaptation to salinity and temperature on the energetic nutrient plus AMP/albumin ratio might bring more data than inhibitor studies to explain how quantitative differences in AMP concentration can be translated at the morphological level.

SUMMARY

The metabolic reactions of the nucleic acids blocked by benzimidazole, 2–6 diaminopurine, 6 mercaptopurine, hydroxyurea, actinomycin D and HPP are essential for *Artemia* growth and survival.

The morphogenetic action of AMP deficiency is not induced by the administration of any of these antagonists. Xanthine dehydrogenase activity has been shown to vary with the quantitative and qualitative nature of the dietary purine derivatives.

At 24‰ salinity maximal protein synthesis estimated by minimal puromycin and parafluorophenylalanine action depends on the AMP and albumin concentrations of the diet and corresponds to a 100:20 ratio.

At 120‰ salinity the optimal AMP:albumin ratio stands at 40:20. Salinity reduces the AMP requirement for maximal protein synthesis. The data presented suggest that AMP concentration controls growth rate and abdominal length through different metabolic systems.

LITERATURE CITED

BEERSTECHER, E., JR., S. CORNYN AND C. VOKMANN, 1954. Invertebrate nutrition. II. The effects of vitamin and amino acid analogues on *Oniscus asellus*. *Tex. Repts. Biol. Med.*, **12**: 212–214.

CROGHAN, P. C., 1958. The mechanism of osmotic regulation in *Artemia salina* (L): the physiology of the gut. *J. Exp. Biol.*, **35**: 243–249.

DAGG, M. J., 1969. Relationship between growth rate and RNA, DNA protein and dry weight in *Artemia salina* and *Euchaeta japonica*. *M.S. Thesis, University Victoria*, Victoria, B. C.

Ewing, R. D., G. L. Peterson and F. P. Conte, 1972. Larval salt gland of *Artemia salina* nauplii. Effects of inhibitors on survival at various salinities. *J. Comp. Physiol.,* **80**: 247–254.

Fautrez-Firlefyn, N., and J. Fautrez, 1970. Effets sur *Artemia salina* d'hydroxyurée introduite dans le milieu. *Biol. Jahr. Belg.,* **38**: 95–101.

Geer, B. W., 1963. A ribonucleic acid-protein relationship in *Drosophila* nutrition. *J. Exp. Zool.,* **154**: 353–364.

Gilchrist, B. M., 1956. The oxygen consumption of *Artemia salina* (L.) at different salinities. *Hydrobiologia,* **8**: 54–65.

Hernandorena, A. 1970. Action de deux inhibiteurs d'enzyme intervenant au niveau du métabolisme des ptérines chez *Artemia salina* L. (Phyllopoda) en milieu axénique. *Crustaceana,* **19**: 49–58.

Hernandorena, A., 1972a. Signification morphogénétique du besoin alimentaire en acides nucléiques chez *Artemia salina.* I. Besoin en thymidine. *Arch. Zool. Expér. Gén. Fr.,* **113**: 425–432.

Hernandorena, A., 1972b. Signification morphogénétique du besoin alimentaire en acides nucléiques chez *Artemia salina.* II. Besoin en dérivés puriques. *Arch. Zool. Exp. Gén.,* **113**: 489–498.

Hernandorena, A., 1972c. Evidence for xanthine dehydrogenase action on lipid metabolism in *Artemia salina* (L.). *Comp. Biochem. Physiol.,* **42A**: 939–944.

Hernandorena, A., 1974a. Effects of salinity on nutritional requirements of *Artemia salina. Biol. Bull.,* **146**: 238–248.

Hernandorena, A., 1974b. Besoin alimentaire en acide adénylique, croissance et morphogénèse d'*Artemia salina. Ann. Nutr. Alim.,* **28**: 65–82.

House, H. L., 1966. Effects of varying the ratio between the amino acids and the other nutrients in conjunction with a salt mixture on the fly *Agrias affinis* (Fall). *J. Insect. Physiol.,* **12**: 299–310.

Ito, T., and F. Mukaiyama, 1964. Relationship between protein content of diets and xanthine oxydase activity in the silkworm *Bombyx mori* L. *J. Insect Physiol.,* **10**: 789–796.

Kato, M., and K. Sumimoto, 1968. Nutritional studies on excessive larval molting in the silkworm *Bombyx mori. Annat. Zool. Jap.,* **41**: 140–147.

Kelley, W. N., 1972. Purine and pyrimidine metabolism in cells in culture. In G. H. Rothblat and V. J. Cristofalo, Eds., *Growth, nutrition and metabolism of cells in culture.* Academic Press, New York.

Mason, D. T., 1963. The growth response of *Artemia salina* (L.) to various feeding regimes. *Crustaceana,* **5**: 138–150.

Provasoli, L., and A. d'Agostino, 1969. Development of artificial media for *Artemia salina. Biol. Bull.,* **136**: 434–453.

Sang, J. H., 1959. Circumstances affecting the nutritional requirements of *Drosophila melanogaster. Ann. N. Y. Acad. Sci.,* **77**: 352–365.

Stirpe, F., and E. Della Corte, 1965. Regulation of xanthine dehydrogenase in chick liver: effect of starvation and of administration of purines and purine nucleosides. *Biochem. J.,* **94**: 309–313.

Villela, G. G., A. M. Calcagnotto, M. C. Piedras Lopes, and M. M. Rios Magalhaes, 1970. Studos sôbre as enzimas do catabolismo das purinas em *Tenebrio molitor* Linn. *An. Acad. Bras. Ci., Brasil,* **42**: 193–198.

Reference: *Biol. Bull.,* **148**: 429–439. (June, 1975)

JUVENILE HORMONE-INDUCED DELAY OF METAMORPHOSIS OF THE VISCERA OF THE CECROPIA SILKWORM[1]

LYNN M. RIDDIFORD

Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138, and Department of Zoology, University of Washington, Seattle, Washington 98195[2]

Metamorphosis in the Lepidoptera begins when the corpora allata are inactivated in the final larval instar and the juvenile hormone (JH) titer subsequently declines (Williams, 1961; DeWilde, de Kort and de Loof, 1971; Nijhout and Williams, 1974). In response to ecdysone in the presence of this small amount of JH, the integument and viscera progress from the larval to the pupal state. The artificial maintenance of a high JH titer at this stage interferes with this transformation (Piepho, 1942; Sehnal, 1968; Sehnal and Meyer, 1968; Riddiford, 1972; Sehnal and Schneiderman, 1973; Truman, Riddiford and Safranek, 1974).

In the epidermis, the switchover from the commitment to larval differentiation to that for pupal differentiation apparently occurs at the time of gut evacuation (Riddiford, 1972; Truman, Riddiford and Safranek, 1974). After that time, in the giant silkworm, *Hyalophora cecropia,* application of JH does not prevent pupal cuticle synthesis and deposition, but it blocks the pupal differentiation of the viscera when given at any time during the prepupal period (Riddiford, 1972).

In this previous study, the state of differentiation of the viscera was ascertained by their response during the adult development which occurred immediately after pupation. Thus, the possibility that sufficient exogenous JH remained in the animal to act directly on the pupal-adult transformation of the viscera was not ruled out. Accordingly, in this investigation, the gonads and fat body from animals treated with JH as prepupae were transplanted immediately after pupal ecdysis to untreated host pupae. In this manner, their competence for adult differentiation could be assayed by their ability to develop in concert with the host pupa.

MATERIALS AND METHODS

Experimental animals

Cecropia larvae were reared on wild cherry trees (Telfer, 1967) or in the laboratory on synthetic medium (Riddiford, 1968). After gut evacuation the animals were maintained at 25–26° C under a 17L:7D photoperiod.

Host pupae were usually in diapause but chilled for varying lengths of time. A few host pupae had had their brains removed to hold them in permanent diapause.

---

[1] This study was supported by grants GB-7966, GB-36645X, and GB-40169X from the National Science Foundation and a grant from the Rockefeller Foundation.

[2] Present address.

Hormonal materials

Cecropia C18-JH (70% all trans) (Eco-Control, Inc.), and its mimics epoxygeranyl sesamole (EGS) (Eco-Control, Inc.), ethyl 3,7,11 trimethyl-dodecadienoate (ZR512) (Zoecon Corporation), and the Williams-Law mixture of chlorinated hydrocarbons (JH-A) prepared according to the method of Vinson and Williams (1967) were used. In the Cecropia pupal assay (Williams, 1961) 0.1 μg C18-JH, 2.5 μg EGS, 15 μg ZR512, or 20 μg JH-A was necessary to give a +3 pupal-adult intermediate.

The juvenile hormone materials were freshly prepared in acetone ("Nanograde," Mallinkrodt), and 2 to 5 μl were applied along the dorsal midline of the prepupa. Alternatively, the hormonal materials were mixed with light mineral oil (Fisher; Saybolt viscosity 125/135), and 50 μl were injected into the prepupa just anterior to the middorsal tubercle on the 8th abdominal segment. 12.5 μg of β-ecdysone (either from K. Sláma or from Rohto Co.) in 50 μl 10% isopropanol were injected into the mesothoracic tergum of a diapausing host pupa to initiate adult development after a specified period of time.

Surgical procedures

The surgical techniques employed were as previously described (Williams, 1952; Schneiderman, 1967). The organs to be transplanted were removed from a larva of a known age or from the donor pupa within 12 hours after pupal ecdysis. They were rinsed in Ringer's (Ephrussi and Beadle, 1936) and all extraneous tissue was removed. Each gonad was placed in the tip of the abdomen of a host pupa of the same sex, and the wound covered by a plastic cover slip. The fat body transplants were always made between diet- and leaf-reared animals since the fat body of the former is white whereas that of the latter is yellow. This color difference persisted through metamorphosis; thus, the implant could be readily identified.

Abdomens were isolated by slicing through the pupa at the level of the posterior mesothoracic tergum. The midgut was thus left intact and allowed to recede into the abdominal compartment as fat body was removed. The pupa was then transected at the level of the first abdominal segment, and the abdomen sealed with a plastic cover slip. The abdomens were stored in an inverted position at 25° C. Several days later, the gonad was implanted either into the top of the isolated abdomen through the paraffin-plugged hole in the cover slip or into the tip of the abdomen. After varying lengths of time at 25° C, the implant was removed and placed into an intact diapausing host pupa to assay its developmental stage. This further operation was necessary because after β-ecdysone injection, few of the isolated abdomens survived long enough to complete adult development.

RESULTS

Effects of JH application at the initiation of the prepupal stage

I previously reported that when JH-A was administered to a Cecropia prepupa at the time of ocellar retraction, the subsequent pupal diapause was averted and the resultant individual was adult externally but pupal internally (Riddiford,

TABLE I

Effects on the incidence of diapause and on adult differentiation of administration of Cecropia C18-JH to Cecropia prepupae at the time of ocellar retraction.

Dosage‡ (μg)	Number treated	Number failing to diapause	Juvenile characters in resulting moths†			
			External*	Internal**		
				Thorax	Fat body	Gonads
Topical application in acetone						
2.5–5	3	0	—	—	—	—
10–15	5	3	0.5	3.1	3.3	3.1
25–50	4	4	1.5	4.1	5.0	4.1
100	2	2	3.2	5.0	5.0	5.0
Injection in light mineral oil						
0.1–1	3	0	—	—	—	—
2.5–5	5	3	0.5	3.0	4.5	4.0
10–25	8	8	1.0	4.2	4.7	4.2
50	3	3	3.0	4.7	4.9	4.4
100	4	4	4.2	4.9	5.0	4.8

‡Doses which produced the same scores were combined; thus, the range is given.

† When there is more than one treated individual, an average score is used. This average is based only on the scores for nondiapausing individuals.

* Scoring of external characteristics according to the 0 to 5 scale of Williams (1961).

** Scoring of internal characteristics according to the 0 to 5 scale of Riddiford (1972).

1972). Since Cecropia C18-JH and more specific JH mimics have become available, a study of their effects at this critical time of pupal development was done in preparation to a study of the effects of JH on the larval-pupal transformation of the viscera.

Table I shows the effects of Cecropia C18-JH on adult differentiation when it is applied to Cecropia prepupae at the time of ocellar retraction. Just as previously found with JH-A (Riddiford, 1972), moderate doses of C18-JH prevented diapause and the metamorphosis of the viscera but had little effect on the integument. With the higher doses of C18-JH, more severe external effects were obtained. Since the resultant pupae had appeared normal externally, these external effects noted in the adult were not due to JH acting at the time of application but rather to the hormone which persisted until the beginning of adult development. As seen in Table I, a given dose of C18-JH was much more effective when injected in mineral oil than when applied topically in acetone. Undoubtedly, this is due to the fact that topically applied hormone is metabolized much faster than that injected in mineral oil (Ajami and Riddiford, 1973; Riddiford and Ajami, 1973).

Similar experiments involving topical application of the JH mimics at the onset on the prepupal period indicated that these compounds were more stable in the animals than was the Cecropia C18-JH since dosages just sufficient to prevent metamorphosis of the viscera also produced external effects in the adult. For

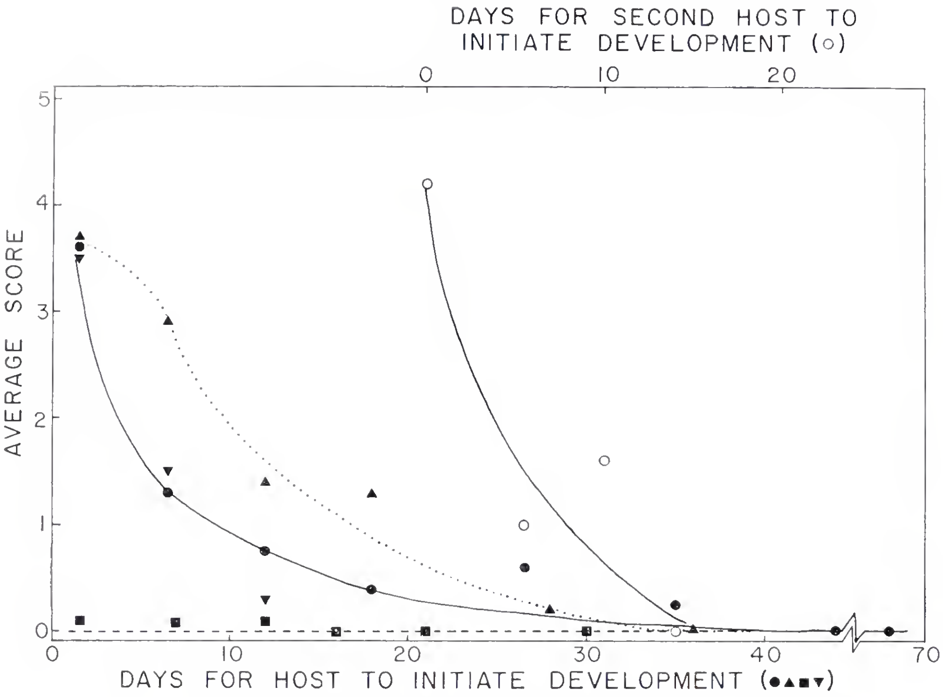


FIGURE 1. The average score of the implanted testes, ovaries, and fat body as a function of the mean time (±1 day) required for the initiation of adult development of the host pupa after the implantation. Implants from: closed squares represent untreated fresh (< 12-hour old) pupae (15 implants per point until day 8; 6 for day 12; and 3 thereafter); closed circles, fresh pupae given 25 μg C18-JH at onset of prepupal period (at least 15 implants per point except 9 for days 0–3 and 4 for days 3–6); triangles, fresh pupae given JH mimics (150 μg JHA, 50 μg ZR512, or 50 μg EGS) at onset of prepupal period (at least 25 implants per point through 15 days, then 12 for days 17–21, and 4 thereafter); inverted triangles, fresh pupae given 50 μg EGS as white prepupae (at least 10 implants per point); open circles, isolated abdomens 14–36 days after removal from fresh pupae treated with 25 μg C18-JH at onset of prepupal period (6 implants per point except only 1 at 14 days).

instance, 30 μg EGS was the minimum dose which prevented diapause, yet treated individuals showed an external score of 3.0 as well as complete inhibition of metamorphosis of the viscera. Similarly, 10 μg ZR512 usually prevented diapause with nearly complete inhibition of internal metamorphosis, but it also partially inhibited the pupal-adult transformation of the integument (3.0 score).

Assessment of the developmental status of viscera from JH-treated pupae

Thus, a low dose of the animal's own juvenile hormone applied at the onset of the prepupal period interfered with metamorphosis of the viscera without affecting the integument. But, as with the previous study with JH-A (Riddiford, 1972), it was possible that the JH was persisting in the animal through the prepupal period and acting primarily at the beginning of adult development. If this were true, the differences in responses of the viscera and of the integument would only indicate that the internal organs were more sensitive to the hormone. Therefore, the developmental status of the viscera at the time of pupal ecdysis was studied by transplanting various organs into chilled diapausing host pupae. Transplants of any part of the gut, the Malphigian tubules, and the nerve cord of the untreated freshly pupated individuals proved largely unsuccessful. But, as seen in Figure 1, all 45 gonad and fat body implants underwent normal adult development in concert with the host irrespective of the time required for the host to initiate adult development. The number of mature eggs produced by the implanted ovary was small (an average of 22 chorionated ones compared to 125 per ovary *in situ*). But since the host's ovaries always made their normal complement of eggs, the space and nutrients available to the third implanted ovary was most likely limited. Therefore, in Table II, a score of 0 is assigned to an ovary which makes at least 12 chorionated eggs. These results indicate that in Cecropia by the time of pupal ecdysis the viscera have completed pupal development and are competent to undergo adult development immediately.

To assess the effect of JH on the larval-pupal transformation of the viscera, prepupae were treated at the onset of ocellar retraction with a dose of C18-JH or a JH mimic that prevented the metamorphosis of the viscera (> + 4 score). Five to six days later within 12 hours of pupal ecdysis the gonads and fat body from the treated animals were transplanted into diapausing host pupae. One day after the subsequent adult emergence of the host, the developmental status of the implant was scored as outlined in Table II.

Figure 1 shows the response of the implant as a function of the lag between the time of implantation and the subsequent onset of adult development of the

Table II

Assessment of developmental status of viscera at time of pupal ecdysis by implantation and subsequent differentiation during adult development of host pupa.

Score†	Testis*	Ovary	Fat body
0	Greater than 90% mature sperm.	At least 12 chorionated** and 12 chorionating eggs.	Adult.
+1	75–90% mature sperm, remainder of stages II, III and IV.	5–11 chorionated eggs and some chorionating follicles.	Adult with trace dissociated fat body.
+2	50–75% mature sperm; remainder of stages II to IV.	Up to 4 chorionated eggs; mainly small vitellogenic follicles.	Mixture of adult and granular type.
+3	Few mature; less than 50% stage IV; remainder stages I–III.	No chorionated eggs; many small vitellogenic follicles.	Granular along trachea, neither pupal nor adult in appearance.
+4	Less than 10% stage IV; mainly stages I and II.	Ovarioles grown out with distinct follicles but little or no yolk deposited.	Friable.
+5	All stage I: pupal.	Pupal.	Pupal.

† 0 indicates that viscera were pupal at time of pupal ecdysis and implantation; +5 indicates that viscera were larval at the time.

* The stages of spermatogenesis are those given by Kambysellis and Williams (1971).

** Average of 22 chorionated and 12 chorionating eggs among the 14 control implants.

host. When the host pupa initiated adult development within three days of implantation, the implants showed only traces of adult characters and responded quite similarly to organs left *in situ* in JH-treated animals. In contrast to the control implants, then, these JH-treated viscera had not completed pupal differentiation by the time of pupal ecdysis so were unable to undergo adult development. But if development of the host pupae was delayed, the implant rapidly became competent to undergo adult development; and by three to four weeks all implants were able to differentiate into adult structures in concert with the host. As seen in Figure 1 the loss of these effects and the acquisition of competence for adult differentiation occurred more rapidly after treatment of prepupae with C18-JH than with the more stable JH mimics. Thus, when treated with JH at the onset of the prepupal period, the viscera are unable to complete the larval-pupal transformation by the time of pupal ecdysis but can complete it in the JH-free environment of the diapausing pupal host.

My previous studies (Riddiford, 1972) had indicated that JH treatment at the white prepupal stage (just before pupal ecdysis) had a definite but less pronounced effect on the metamorphosis of the viscera. In this instance, the effect might not be on the larval-pupal transformation of viscera which had been in progress for 3 to 4 days at the time of application but rather only on their pupal-adult transformation which began about 2 to 3 days after application. In order to differentiate between these two possibilities, white prepupae were treated with 50 μg EGS, and the gonads and fat body subsequently removed about 26 hours later within 12 hours of pupal ecdysis. As seen in Figure 1, when the host pupae immediately initiated adult development, the implants showed little adult differentiation, similar to those from animals treated with EGS at the outset of pupal development. But the time necessary for recovery was much faster as would be expected since the larval-pupal transformation was nearly complete at the time of JH application.

Role of brain and prothoracic glands in pupal development of JH-treated viscera

The above results clearly indicated that the JH-blocked viscera could gain competence to undergo adult development when they were implanted into diapausing pupae. To determine if this recovery was influenced by the endocrine environment of the host, the effects of brain and prothoracic gland removal were examined.

For assay of the role of the brain, brains were removed from the diapausing pupae. The organ from JH-treated prepupae was removed immediately after pupal ecdysis and implanted into the brainless host; then β-ecdysone was injected at specific times thereafter. The time of recovery of the implant in a debrained pupa was found to be the same as that for an intact host. Therefore, the data in Figure 1 are a composite of implants into hosts with and without brains.

To assess the effects of the prothoracic glands on the recovery of the viscera, gonads were removed from the JH-treated individuals and placed into isolated diapausing pupal abdomens. After 14, 21, or 36 days the implants were removed and implanted into chilled diapausing host pupae to assay their developmental status. In no instance did the gonad undergo adult differentiation when adult development of the host began immediately. In fact, the developmental status of

TABLE III

Developmental capacity of larval gonads after varying lengths of time in a diapausing pupa.

Stage of donor	Time for host to initiate development	Number implants	Developmental capacity of implant average score ±s.d.
4th instar	1–3 days	4	4.8 ± 0.5
(1–2 g)	$3\frac{1}{2}$ months*	3	2.7 ± 1.5
5th instar	1–3 days	5	5.0 ± 0.0
(2–8 g)	5–8 days	4	4.5 ± 0.6
	10–14 days	6	3.2 ± 0.9
	$3\frac{1}{2}$ months*	7	1.2 ± 1.4
Gut evacuation	5–8 days	1	4.0
	$3\frac{1}{2}$ months*	2	0.5 ± 0.7

* Brainless host pupae injected with 12.5 μg β-ecdysone $3\frac{1}{2}$ months after implantation.

the implant had not changed during the 14 to 36 days in the isolated abdomens. In Figure 1 all implants from isolated abdomens are grouped together as if they had been transplanted into the chilled host after 21 days. The data show that the treated gonads did not become competent to undergo adult development until after they were placed into the intact pupa. Then the kinetics of recovery was essentially the same as that seen after implantation of the treated gonad immediately after pupation. The average score of 1.6 at 12 days is for 6 implants, 4 of which formed normal adult structures whereas both ovaries from one treated individual remained pupal. Apparently then the presence of prothoracic glands was essential for the completion of the pupal transformation after the JH effects had decayed.

Capacity of larval viscera for adult differentiation

Since the hormonal environment of an intact or brainless diapausing pupa was sufficient to allow organs from JH-treated prepupae to attain competence for adult differentiation, it was of interest to determine whether organs from feeding larvae could also undergo the pupal transformation in a diapausing pupal host.

The gonads of 4th and 5th instar larvae of known age were transplanted into diapausing host pupae. Table III shows that when the host pupa initiated adult development within 8 days after implantation, the larval gonad was not capable of undergoing adult development. When development of the host was delayed for 10 to 14 days, then the implant showed some adult development forming a few mature sperm or oocytes but remained less than half normal adult size. After $3\frac{1}{2}$ months in a brainless diapausing host pupa, the testis was able to mature fully in spite of its small size. The ovary was able to form some chorionated eggs and at least an equal number of vitellogenic follicles but many fewer than implants from freshly ecdysed pupae. The lack of the period of growth of the ovary which normally occurs during the larval-pupal transformation may account for the fewer developing follicles and could explain the disparity between the developmental capacities of the ovary and the testis. These experiments however clearly indicate that when placed in a JH-free environment under the influence of the prothoracic glands, the larval gonad can undergo pupal development without the concomitant growth that normally occurs during the larval-pupal transformation.

DISCUSSION

During larval life the gonads grow slowly. Then when the JH titer declines and metamorphosis begins, they show an increased rate of growth and an initial differentiation prerequisite for subsequent adult development (Sehnal, 1968). The results obtained here show that gonads from early final instar larvae can be induced to make adult structures in response to ecdysone after being held in a diapausing host pupa for several weeks. During this holding period, conditions in the host pupa were apparently suitable for these organs to undergo their initial pupal differentiation. Since these larval implants never attained normal adult size, it is obvious that the period of enhanced growth is not necessary for their larval-pupal transformation.

This initial differentiation of the viscera to the pupal condition requires ecdysone in the presence of little or no JH (Williams, 1961). Juvenile hormone treatment of the prepupa prevented pupal differentiation, and treated viscera showed no recovery after 2 to 5 weeks in isolated abdomens. But after reimplantation into intact pupae, pupal differentiation occurred during the ensuing two weeks before adult development was initiated, and the implants completed adult differentiation in concert with their hosts. Since recovery of treated implants occurred at the same rate, irrespective of whether or not the host pupa had a brain, this process was undoubtedly due to ecdysone release from the prothoracic glands.

Normally, the prothoracic glands of Cecropia secrete sufficient ecdysone to initiate molting only after activation by the prothoracicotropic hormone from the brain (Williams, 1952). But wounding can subliminally activate these glands in Cecropia, and in other saturniid species can fully activate them (McDaniel and Berry, 1967). Furthermore, debrained tobacco hornworm (*Manduca sexta*) pupae can nevertheless eventually develop (Judy, 1972). Therefore, it seems likely that a small amount of ecdysone may "leak out" of the prothoracic glands in the wounded diapausing pupal hosts. While this ecdysone is insufficient to trigger adult development, it is sufficient for the completion of pupal differentiation of the implant. This amount of ecdysone also allows pupal differentiation of the larval gonad but appears inadequate to induce the increased growth rate normally seen at the outset of metamorphosis.

The recovery of the JH-treated viscera in host pupae can be divided into three phases: 1) the decay of the exogenous hormone; 2) the decay of the covert effects of the hormone; and 3) the subsequent completion of the larval-pupal transformation. When the third phase is completed, the pupal viscera are able to respond to ecdysone in the absence of JH and become adult. Similar recovery after JH treatment was noted by Sehnal and Schneiderman (1973) in the waxmoth, *Galleria mellonella*.

The decay of exogenous hormone is quite rapid—the half-life of Cecropia C18-JH in the blood is at most two hours (Ajami and Riddiford, 1973). The half-life of the JH mimics is somewhat longer (Staal, 1975) and could be seen from their effects on the integument when adult development began about 10 days after application. Thus, the organs from JH-mimic treated prepupae were exposed to a higher level of JH at the time of their removal and consequently, as seen in Figure 1, required a longer time to attain competence for adult differentiation. It is interesting in this context that only a rare host with an implant formed pupal cuticle at the wound site indicating that the implant *per se* did not contain significant amounts of unbound hormone.

The separation of the last two phases of recovery—the decay of covert effects and the completion of larval-pupal differentiation—is more difficult. One difference between the two is that the latter requires the presence of ecdysone whereas the former apparently does not. Kimura (1974) and Fain and Riddiford (1973) have shown that the covert effects of JH that are prerequisite for a larval molt have completely decayed by 48 hours and 72 hours in isolated abdomens of *Bombyx mori* and *Manduca sexta* respectively. Thus, after 2 to 5 weeks in an isolated abdomen the Cecropia implants were presumably free of exogenous JH and its covert effects. The subsequent time then required for these organs to recover after implantation into diapausing hosts must reflect the time needed to complete differentiation to the pupal condition. As seen in Figure 1, this time is only slightly shorter than that required by implants taken directly from ^{18}C-JH-treated individuals. Thus, the recovery rates indicated by the curves in Figure 1 reflect primarily the time required for the completion of pupal differentiation after the effects of juvenile hormone have disappeared. Although normally pupal differentiation in Cecropia requires 4 to 5 days (Williams, 1952), the increased time found necessary in these experiments likely is due to the very low level of ecdysone in the diapausing hosts.

Presumably in Cecropia as in *Manduca* (Bollenbacher, Vedeckis, Gilbert and O'Connor, 1975) there is a high titer of ecdysone at the onset of the prepupal period. This large amount apparently is necessary to initiate pupal cuticle synthesis and also probably accounts for the growth of the gonads. The experiments reported here, however, indicate that pupal differentiation of the viscera can occur in the presence of much lower amounts of ecdysone. But this differentiation of the viscera requires the virtual absence of JH throughout the prepupal period. In the epidermis, only the commitment to pupal differentiation which occurs in response to the first ecdysone release that initiates gut evacuation (Truman and Riddiford, 1974; Bollenbacher *et al.*, 1975) can be prevented by JH (Riddiford, 1972; Truman, Riddiford and Safranek, 1974). After this time epidermal differentiation becomes aloof to JH. Thus, the epidermis and viscera differ not only in the timing of their irrevocable switchover from larval to pupal commitment, but also in the amount of ecdysone necessary for the expression of that commitment.

I thank Saundra Troisi and Angela Ng for rearing the Cecropia; Dr. Alfred Ajami for providing the Cecropia C18-juvenile hormone and its mimic EGS; Dr. John Siddall for the mimic ZR512; Professor Carroll M. Williams for the JH-A, and Dr. Karel Sláma for the β-ecdysone; Professor James Truman for help with the abdominal isolation technique and valuable criticisms during the preparation of this manuscript; and Professor John Edwards and Ms. Mary Nijhout for a critical reading of the manuscript.

SUMMARY

1. Cecropia C18-juvenile hormone (C18-JH) when given to Cecropia larvae at the onset of pupal development prevented metamorphosis of the viscera but had

little effect on the integument as had been previously reported for a mixture of JH mimics (Riddiford, 1972).

2. The developmental status of the viscera of freshly ecdysed pupae which had been treated with juvenile hormone (C18-JH or JH mimics) as prepupae was ascertained by transplantation into normal host pupae.

3. Recovery as signaled by the completion of the larval-pupal transformation of these implanted viscera occurred in diapausing pupae with or without brains but not in isolated pupal abdomens. Thus, ecdysone is necessary for the resumption of differentiation after JH and its effects have decayed.

4. Similarly, pupal differentiation of larval gonads occurred during 3½ months in a diapausing host pupa. Thus, for pupal differentiation of the viscera all that is required is a low level of ecdysone in the absence of JH.

5. The epidermis and the viscera thus differ in their hormonal requirements for pupal differentiation. The epidermis requires only an absence of JH during the time of commitment but not during its expression; furthermore, this expression requires a high level of ecdysone.

LITERATURE CITED

Ajami, A. M., and L. M. Riddiford, 1973. Comparative metabolism of the Cecropia juvenile hormone. *J. Insect Physiol.*, **19**: 635–645.

Bollenbacher, W. E., W. V. Vedeckis, L. I. Gilbert, and J. D. O'Connor, 1975. Ecdysone titers and prothoracic gland activity during the larval-pupal development of *Manduca sexta*. *Develop. Biol.*, in press.

DeWilde, J., C. A. D. de Kort, and A. de Loof, 1971. The significance of juvenile hormone titers. *Mitt. Schweiz. Entomol.*, **44**: 79–86.

Ephrussi, B., and G. W. Beadle, 1936. A technique of transplantation for *Drosophila*. *Am. Nat.*, **70**: 218–225.

Fain, M. J., and L. M. Riddiford, 1973. *In vivo* and *in vitro* response of larval crochet epidermis to ecdysone and juvenile hormone. *Am. Zool.*, **13**: 1272.

Judy, K. J., 1972. Diapause termination and metamorphosis in brainless tobacco hornworms (Lepidoptera). *Life Sci.*, **11(2)**: 605–611.

Kambysellis, M. P., and C. M. Williams, 1971. *In vitro* development of insect tissues. I. A macromolecular factor prerequisite for silkworm spermatogenesis. *Biol. Bull.*, **141**: 527–540.

Kimura, S., 1974. Relationship between hormone titres and RNA and protein synthesis when the change to the pupal programme occurs in the silkworm, *Bombyx mori*. *J. Insect Physiol.*, **20**: 887–895.

McDaniel, C. N., and S. J. Berry, 1967. Activation of the prothoracic glands of *Antheraea polyphemus*. *Nature*, **214**: 1032–1034.

Nijhout, H. F., and C. M. Williams, 1974. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L): cessation of juvenile hormone secretion as a trigger for pupation. *J. Exp. Biol.* **61**: 493–501.

Piepho, H., 1942. Untersuchungen zur Entwicklungsphysiologie der Insektenmetamorphose. Über die Puppenhautung der Wachsmotte *Galleria mellonella L. Wilhelm Roux' Arch. Entwicklungsmech. Organismen*, **141**: 500–583.

Riddiford, L. M., 1968. Artificial diet for Cecropia and other saturniid silkworms. *Science*, **160**: 1461–1462.

Riddiford, L. M., 1972. Juvenile hormone in relation to the larval-pupal transformation of the Cecropia silkworm. *Biol. Bull.*, **142**: 310–325.

Riddiford, L. M., and A. M. Ajami, 1973. Juvenile hormone: its assay and effects on pupae of *Manduca sexta*. *J. Insect Physiol.*, **19**: 749–762.

Schneiderman, H. A., 1967. Insect Surgery. Pages 753–766 in F. H. Wilt and N. K. Wessells, Eds., *Methods in Developmental Biology*. T. Y. Crowell Co., New York.

SEHNAL, F., 1968. Influence of the corpus allatum on the development of internal organs in *Galleria mellonella* L. *J. Insect Physiol.*, **14**: 73–85.

SEHNAL, F., AND A. S. MEYER, 1968. Larval-pupal transformation: control by juvenile hormone. *Science*, **159**: 981–983.

SEHNAL, F., AND H. A. SCHNEIDERMAN, 1973. Action of the corpora allata and of juvenilizing substances on the larval-pupal transformation of *Galleria mellonella* (Lepidoptera). *Acta Entomol. Bohemoslav.*, **70**: 289–302.

STAAL, G. B., 1975. Insect growth regulators with juvenile hormone activity. *Ann. Rev. Entomol.*, **20**: 417–460.

TELFER, W. H., 1967. Cecropia. Pages 173–182 in F. H. Wilt and N. K. Wessells, Eds., *Methods in Developmental Biology.* T. Y. Crowell Co., New York.

TRUMAN, J. W., AND L. M. RIDDIFORD, 1974. Physiology of insect rhythms. III. The temporal organization of the endocrine events underlying pupation of the tobacco hornworm. *J. Exp. Biol.*, **60**: 371–382.

TRUMAN, J. W., L. M. RIDDIFORD, AND L. SAFRANEK, 1974. Temporal patterns of response to ecdysone and juvenile hormone in the epidermis of the tobacco hornworm, *Manduca sexta*. *Develop. Biol.*, **39**: 247–262.

VINSON, J. W., AND C. M. WILLIAMS, 1967. Lethal effects of synthetic juvenile hormone on the human body louse. *Proc. Nat. Acad. Sci. U. S.*, **58**: 294–297.

WILLIAMS, C. M., 1952. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm. *Biol. Bull.*, **103**: 120–138.

WILLIAMS, C. M., 1961. The juvenile hormone. II. Its role in the endocrine control of molting, pupation, and adult development in the Cecropia silkworm. *Biol. Bull.*, **121**: 572–585.

Reference: *Biol. Bull.*, **148**: 440–447. (June, 1975)

SOME EFFECTS OF EYESTALK REMOVAL ON THE Y-ORGANS OF *CANCER IRRORATUS* SAY

FRANK P. SIMIONE, JR.[1] AND DANIEL L. HOFFMAN

Department of Biology, Bucknell University, Lewisburg, Pennsylvania 17837

Brachyuran crustaceans possess a pair of endocrine glands called Y-organs which are thought to be homologous to the molting glands of insects (Gabe, 1953). Bilateral removal of these Y-organs prevents molting in *Carcinus maenas* (Echalier, 1954) and *Sesarma reticulatum* (Passano and Jyssum, 1963); and reimplantation leads to the resumption of the normal molting process (Echalier, 1955). Bilateral eyestalk removal in the fiddler crab, *Uca pugilator* (Abramowitz and Abramowitz, 1940), has been shown to lead to precocious molting when it is performed during the intermolt period of the molt cycle, but not when performed during the premolt period in *Sesarma reticulatum* (Passano and Jyssum, 1963). It appears that the decapod crustacean eyestalk neurosecretory complex secretes a hormone that inhibits the Y-organ from producing a molting hormone (Passano, 1960; Charniaux-Cotton and Kleinholz, 1964). Eyestalk removal or destalking appears to result in the hypertrophy of Y-organ cells as evidenced in *Hemigrapsus nudus* (Matsumoto, 1962) and *Libinia emarginata* (Hinsch, 1973). Yet an important consideration is whether or not this hypertrophy is indicative of cellular activity. One method of monitoring Y-organ activity is quantification of RNA synthesis as measured by the uptake of a radioactive precursor, ^{3}H-uridine (Comings, 1966; Brasiello, 1968; Gorell and Gilbert, 1969; Foulks and Hoffman, 1974). The following experiment was designed to test whether there is any statistically significant difference in the incorporation rate of ^{3}H-uridine into the Y-organ cells of a crab at various time periods following bilateral eyestalk removal.

MATERIALS AND METHODS

Female specimens of the East Coast rock crab, *Cancer irroratus* Say, were obtained from Sheepscot Specimen Company, West Southport, Maine. The animals were maintained in a 25 gallon Instant Ocean Culture System at 10° to 11° C and 35 to 37 ppt salinity. The crabs were regularly fed fish fillets prior to the beginning of the experiments. The destalking experiments were undertaken from October 7th through December 7th, 1973; and the 75 animals, all non-ovigerous, that were used in these experiments ranged in weight from 40 to 148 g.

Both eystalks were removed by severing them at their bases with a fine pair of scissors. The wounds were not cauterized and the crabs were allowed to rest 12 hours to recover from the trauma of the surgery. A control group of animals (Group 1) was not destalked. There were five groups of bilaterally destalked animals, Groups 2 through 6. These animals were destalked 1.5, 2.5, 4.5, 7.5 and 10.5 days, respectively, prior to being injected with the labeled uridine. Both control and destalked crabs were injected with aqueous solution of ^{3}H-uridine using a one ml tuberculin syringe into the arthrodial membrane at the base of the 4th or 5th left walking leg. The given dose was 0.25 microcuries/g weight animal. The ^{3}H-uridine (NET 174, New England Nuclear, Boston, Massachusetts) had specific activities ranging from 26.1 to 27.8 Ci./mM. The wound was cauterized immediately after injection. All animals were sacrificed 24 hours post injection. Thus the time interval after destalking in which uridine incorporation was measured is one day later than the injection time, *i.e.*, 2.5, 3.5, 5.5, 8.5 and 11.5 days postdestalking. Using the amount of deposition of new cuticle at the time of sacrifice (Passano, 1960), all the control and experimental animals were found to be in premolt.

---

[1] Present address: American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

A glutaraldehyde fixation was used for epoxy sections. Primary fixation in glutaraldehyde and postfixation in osmium are outlined in Hoffman (1969). The tissue was embedded in epoxy resins according to the method of Luft (1961). Thick sections approximately 0.5 to 1.5 microns were made for autoradiographs and for microscopic examination. In the latter case, thick sections were stained with Richardson's stain (Richardson, Jarett and Finke, 1960).

Autoradiographs were prepared by dipping unstained slides into a 50% aqueous solution of Kodak NTB-3 liquid emulsion, and exposing them for 4 weeks at 5° C. They were developed and fixed according to the method outlined by Bogoroch (1972). The autoradiographs were examined using phase contrast microscopy with an oil immersion lens and an ocular with a calibrated grid. The grid consisted of 100 squares each measuring 10 microns on a side at 1000 X. Counts of reduced silver grains were undertaken over five randomly selected areas of each Y-organ. In each of these areas, silver grains were counted in five randomly selected grid squares, giving a total of 500 square microns of tissue area counted. The mean silver grain count for the five different areas of each gland was then determined. Background counting was done in the same manner over areas of epoxy resin without tissue. Background counts were subtracted from the counts made over the tissue. Cell boundaries could not be easily distinguished for direct measurements of the cells. Relative cell sizes in the Y-organs were determined by counting the number of nuclei per 500 square microns of tissue using the same counting technique as that used for counting the reduced silver grains.

Statistical analysis was performed on the differences between the means of the various experimental groups. Wherever significant F values were found using an analysis of variance, significance was tested between groups using multiple t-tests. Since the experimental groups contained unequal numbers of glands further testing was done using the Scheffé test (Scheffé, 1959).

Six Y-organs taken from animals that had been destalked for 2.5 days and then injected with an equivalent dose of ^{3}H-uridine were incubated in RNAase with Millonig's phosphate buffer at 37° C for 1 hour in order to test whether the label was being incorporated into RNA. The mean silver grain counts for these Y-organ sections (4.00 ± 1.43 S.E.) were statistically below the average mean count for the group destalked for 2.5 days (12.14 ± 1.04 S.E.) at $P < 0.05$. Therefore, the concentrated incorporation of ^{3}H-uridine into Y-organ cells was RNAase sensitive and was considered evidence of RNA synthesis.

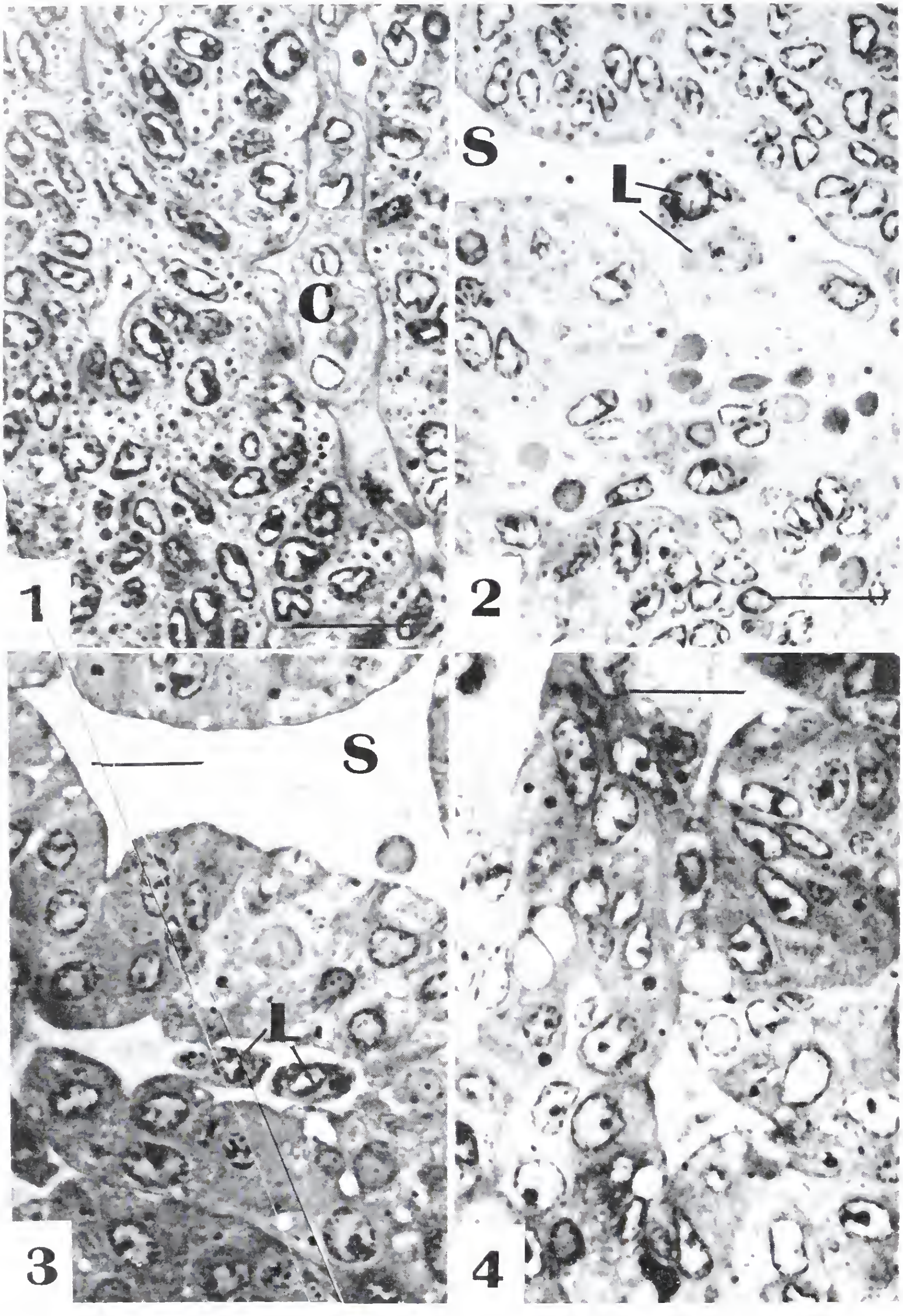


FIGURE 1. Thick epoxy section (1 μ) through the Y-organ of a control (non-destalked) female specimen of *Cancer irroratus*. Note the numerous osmophilic granules within the

RESULTS

The Y-organs of *Cancer irroratus* are found in the same anatomical location as the Y-organs of *Carcinus maenas* as described by Echalier (1959), and they conform to the description of the classical Y-organ of Gabe (1953). They are paired structures, approximately 1 to 2 mm in diameter, light milky yellow in color, that are located in the cephalothorax anterior to the branchial chamber just posterior and immediately lateral to the eyestalks. The glands are in intimate contact with connective and lymphogenous tissue and directly applied to the hypodermis of the ventral carapace at the intersection of two skeletal ridges. These ridges form the insertion of a small accessory mandibular muscle, the "petit muscle lateral" of Echalier (1959) under which lies the Y-organ.

The histology of the Y-organ

The Y-organ consists of anastomosing cords of epithelial cells separated by numerous interconnected hemocoelic sinuses. Numerous fine capillaries are also evident within these sinuses. The cells have irregularly shaped nuclei approximately 8–10 microns in diameter with peripheral chromatin granules and peripheral or centrally located nucleoli. Mitotic activity was not evident in any of the cells. The cytoplasm contains numerous osmophilic granules that can be quite variable in size, some exceeding one micron in diameter (Fig. 1). In glutaraldehyde-fixed tissue that had not been postfixed with osmium tetroxide, these granules appear yellow in color under phase contrast microscopy. The yellowish color of the gland may be due to these granules since no pigment cells are evident in or on the surface of the gland. The cytoplasm of the cells can be quite variable, ranging from very homogeneously staining with Richardson's stain to highly vacuolated. Cellular limits are not readily evident with light microscopy, but the cells appear to range in size from 15 to 20 microns.

The peripheral areas of the gland show evidence of cellular degeneration (Fig. 2). The nuclei of these degenerating cells are ovoid in shape, and many appear pycnotic staining intensely with Richardson's stain. The cytoplasm of these cells is highly vacuolated containing in addition numerous osmophilic granules. In a few of the glands, peripheral cords of cells appear to be rupturing. This is especially evident in regions of cellular degeneration.

---

vacuolated cytoplasm of the cells. Also note the small capillaries (C) within the blood sinuses between the cords of cells; Richardson's stain. The slash mark represents 25 microns.

FIGURE 2. Thick epoxy section (½ μ) through the Y-organ of a control (non-destalked) female specimen of *Cancer irroratus*. Note the area of degeneration with pycnotic nuclei at the center. Also the lymphocytes (L) can be seen within the blood sinus (S); Richardson's stain. The slash mark represents 25 microns.

FIGURE 3. Thick epoxy section (1 μ) through the Y-organ of a 3.5 day destalked female specimen of *Cancer irroratus*. Note the hypertrophied cells and the region of cytoplasmic vacuolization. The granular lymphocytes (L) are within a blood sinus (S); Richardson's stain. The slash mark represents 25 microns.

FIGURE 4. Thick epoxy section (1 μ) through the Y-organ of an 8.5 day destalked female specimen of *Cancer irroratus*. The cells with vacuolated cytoplasm can be easily differentiated from those with non-vacuolated cytoplasm; Richardson's stain. The slash mark represents 25 microns.

TABLE I

Mean number of nuclei and reduced silver grains counted in control and bilaterally destalked animals.

Group	Number of glands	Time after destalking* (days)	Mean number of nuclei counted ± standard error**	Mean number of reduced silver grains counted ±standard error**
1°	19	0	9.07 ± 0.48	3.21 ± 0.69
2	16	2.5	7.44 ± 0.37	6.38 ± 1.21
3	17	3.5	5.05 ± 0.32	12.14 ± 1.04
4	17	5.5	5.46 ± 0.25	0.18 ± 0.56
5	18	8.5	6.07 ± 0.29	2.74 ± 0.53
6	16	11.5	6.19 ± 0.29	4.52 ± 1.38

* Time of sacrifice, 24 hrs after injection.
** Per 500 sq. microns of gland.
° Control animals, not destalked.

Sparsely scattered irregularly shaped granular lymphocytes are evident within the blood sinuses that separate the cords of Y-organ cells (Figs. 2 and 3). These blood cells are approximately 15 to 30 microns in diameter. The cytoplasm in many of these lymphocytes are densely filled with granules as to obscure the nucleus; whereas in others few granules are present. The granules are osmophilic and are quite variable in size as is the case of the granules that are evident in the Y-organ cells. Arthropod lymphocytes are known to be phagocytic (Lochhead and Lochhead, 1941); however, there is no evidence of phagocytosis or any other association between the Y-organ cells and the lymphocytes.

Cytological effects of eyestalk removal on the Y-organ

The Y-organ cells of bilaterally destalked animals show evidence of hypertrophy, the nuclei appearing larger and more ovoid in shape (Figs. 3 and 4). Because of the increase in cytoplasmic volume regions of degeneration and cytoplasmic vacuolization are more evident. Osmophilic granules appear within the cells; the numbers of such cytoplasmic granules is quite variable from cell to cell. Table I gives the mean number of nuclei counted for all glands including those from animals not bilaterally destalked. The Y-organs from the control group of animals, not bilaterally destalked, show a significantly greater number of nuclei per area counted, at $P < 0.05$, than the Y-organs from animals that have been bilaterally destalked. Tissue sections of Y-organs from crabs that have been destalked for 2–3 days (Group 2) demonstrate a significantly higher number of nuclei than those destalked for 3–4 days (Group 3) and 5–6 days (Group 4), but do not differ significantly from the last two experimental groups of glands. A smaller number of nuclei present in a unit area of gland can be an indication of greater cytoplasmic volume or cell hypertrophy. Therefore, it can be assumed that cellular hypertrophy increases steadily up to four to five days following bilateral destalking and then reaches a plateau not to return to the original condition even after 11 to 12 days after eyestalk removal.

The uptake of ^3H-uridine by Y-organ cells following bilateral eyestalk removal

Table I also illustrates the result of reduced silver grain counts made over randomly selected areas of Y-organs in each group of destalked and control animals. Y-organs from animals that were destalked for 3 to 4 days (Group 3) show the highest number of reduced silver grains counted; and the count in these glands is significanlty higher, at $P < 0.05$, than in all other glands counted. Y-organs from animals destalked for 2–3 days (Group 2) differ significantly only from those in Groups 3 and 4, but are significantly higher in the number of silver grains counted than the control glands (Group 1) and from those glands taken from animals in Groups 5 and 6. Glands from animals destalked for 11–12 days (Group 6) differ significantly from those in Group 4 but not from those in Group 5. There appears to be a gradual increase in the number of reduced silver grains counted up to 3 to 4 days following destalking. The level of ^{3}H-uridine incorporated after 5 to 6 days drops below the level incorporated in the control glands. By day 8–9 (Group 5) the level of uptake measured by silver grain counts approaches that in the control glands, and remains at that level even up to 11–12 days after eyestalk removal.

Discussion

Although cellular hypertrophy in the Y-organ has been observed in the Y-organ following bilateral eyestalk removal (Matsumoto, 1962; Hinsch, 1973), such hypertrophy in *Cancer irroratus* reaches its maximum level within the first four days after eyestalk removal. Following this increase in cell size, the Y-organ cells gradually decrease in size, reaching a level that remains slightly above that of the controls even after 11 to 12 days postdestalking. Paralleling the hypertrophy of the cells, there is also demonstrated an increase in the rate of RNA synthesis. This is demonstrated by the fourfold increase in the uptake of ^{3}H-uridine into the RNA of the Y-organ cells during the first four days postdestalking. Although the nature of the RNA is unknown from these experiments, it is apparent that some inhibition on RNA synthesis has been lifted by the removal of both eyestalks. The decrease in the uptake of the label five to six days postdestalking is not completely understood. It may be an indication of a certain secretory phase in the cycle of the gland. However, it is not a function of the type nor the condition of the labeled precursor that was injected into the animals; for the animals that comprised this experimental group (Group 4) received injections of labeled uridine from the same sample as those in the preceding group (Group 3). The Y-organ may be adapting to the absence of the inhibition from the eyestalk complex since the rate of RNA synthesis approximately has returned to the level of uptake demonstrated by the control animals after nine to twelve days postdestalking.

Madhyastha and Rangneker (1972) have described two cell types in the Y-organ of the crab, *Varuna litterata*. The most abundant cell type is a small epithelial cell consistent with that of *Cancer irroratus*. However, the second cell type is described as containing basophilic cytoplasmic granules and is much rarer in occurrence. It appears that this second cell type may be representative of granular lymphocytes which, in *Cancer irroratus*, can be found within the hemocoelic sinuses of the Y-organ. When the cellular cords are very compact, the lymphocytes become compressed against them and appear to contribute to the histology of the Y-organ.

The areas of cytoplasmic vacuolization and cell degeneration in the Y-organ of *Cancer irroratus* may be evidence of its mode of secretion. Charniaux-Cotton, Zerbib and Meusy (1966) have reported such degenerate areas in the androgenic glands of crustaceans and state that they are evidences of holocrine secretion. Such described areas in the Y-organ of *Cancer irroratus* may also give evidence for holocrine activity. However, no mitotic figures were evident within the glands of *C. irroratus;* but Hoffman (unpublished observations) has noted mitotic activity within the Y-organ cells of destalked specimens of *Cancer productus.* These mitotic figures appear to be in proximity to cells with cytoplasmic vacuolization. Bressac (1973) has observed numerous lysosomal-type inclusion bodies in the Y-organ cells of *Pachygrapsus marmoratus* in electron micrograph studies. The Y-organ cells of *Cancer irroratus* of both control and destalked animals show a variability in cytoplasmic granulation. Although no quantitative measurements were taken of these granules, they appear to be more numerous in cells that contain vacuolated cytoplasm. Novikoff (1969, 1960) has noted large lysosmes or cytolysosomes in dying liver cells and the dying cells of atretic ovarian follicles. Also Scharrer (1966) has reported large irregular membrane bound bodies up to 4 microns in diameter within the degenerating prothoracic gland cells of *Leucophaca* and *Blaberus* a few days after the final molt. Scharrer believes that these bodies represent autophagic vacuoles. However, Fawcett, Long and Jones (1969) have also described cytoplasmic osmophilic granules in steroid secreting cells and indicate that these granules may be lipid droplets. Similar type granules in the Y-organ cell may give credence to the steroidogenic nature of the gland. Recently Bollenbacher and O'Connor (1973) have isolated α-ecdysone from the Y-organs of *Pachygrapsus crassipes* cultured *in vitro.* Yet, before any more definite correlations can be made concerning the precise relationship between the Y-organ and the method of synthesis and release of the crustacean molting hormone, more histochemical and biochemical data are needed, especially from a wide variety of crustaceans.

We wish to thank Ruth McDiffett and Frederick Swarts for their assistance with the statistical analysis.

SUMMARY

1. Bilateral destalking of *Cancer irroratus* increases the rate of RNA synthesis in the Y-organ cells during the first four days postdestalking.

2. The cytoplasmic volume of the Y-organ cells also increases during the first four to five days postdestalking and then drops to a plateau that is slightly above the volume of cells from non-destalked control animals.

3. Numerous areas of cytoplasmic vacuolization and cellular degeneration are evidenced within the control and destalked crabs. It is postulated that this may be evidence of holocrine activity.

4. The Y-organ cells of *Cancer irroratus* contain numerous osmophilic granules both in control and destalked animals.

LITERATURE CITED

Abramowitz, R. K., and A. A. Abramowitz, 1940. Moulting, growth and survival after eyestalk removal in *Uca pugilator*. *Biol. Bull.*, **78**: 179–188.

Bogoroch, R., 1972. Liquid emulsion autoradiography. Pages 66–94 in P. B. Gahan, Ed., *Autoradiography for Biologists.* Academic Press, New York.

Bollenbacher, W. E., and J. D. O'Connor, 1973. Production of an ecdysone by crustacean Y-organs *in vitro*. *Amer. Zool.*, **13**: 1274.

Brasiello, A. R., 1968. Autoradiographic study of ribonucleic acid synthesis during spermatogenesis of *Asellus aquaticus* (Crustacea, Isopoda). *Exp. Cell Res.*, **53**: 252–260.

Bressac, C., 1973. Données sur l'ultrastructure de la glande de mue (organe Y) du crabe *Pachygrapsus marmoratus* (Fabricius). *C. R. Acad. Sci. Paris*, **277**: 1165–1167.

Charniaux-Cotton, H., and L. H. Kleinholz, 1964. Hormones in invertebrates other than insects. Pages 135–198 in G. Pincus, K. Thimann and E. B. Astwood, Eds., *The Hormones, Volume IV.* Academic Press, New York.

Charniaux-Cotton, H., C. Zerbib and J. J. Meusy, 1966. Monographie de la glande androgène des Crustacés supérieurs. *Crustaceana*, **10**: 113–136.

Comings, D. E., 1966. Incorporation of tritium of H-5-uridine into DNA. *Exp. Cell Res.*, **41**: 677–681.

Echalier, G., 1954. Recherches expérimentales sur le rôle de "l'organe Y" dans la mue de *Carcinus moenas* (L.) Crustacé Décapode. *C. R. Acad. Sci. Paris*, **238**: 523–525.

Echalier, G., 1955: Rôle de l'organe Y dans le déterminisme de la mue de *Carcinides* (*Carcinus*) *moenas* (L.) (Crustacés Décapodes); Expériences d'implantation. *C. R. Acad. Sci. Paris*, **240**: 1581–1583.

Echalier, G., 1959. L'organe Y et le déterminisme de la croissance et de la mue chez *Carcinus moenas* (L.) Crustacés Décapode. *Ann. Sci. Natur. Zool. Biol. Anim.*, **1**: 1–57.

Fawcett, D. W., J. A. Long and A. L. Jones, 1969. The ultrastructure of endocrine glands. *Recent Progr. in Hormone Research*, **25**: 315–380.

Foulks, N. B., and D. L. Hoffman, 1974. The effects of eyestalk ablation and β-ecdysone on RNA synthesis in the androgenic glands of the protandric shrimp, *Pandalus platyceros* Brandt. *Gen. Comp. Endocrinol.*, **22**: 439–447.

Gabe, M., 1953. Sur l'éxistence, chez quelques Crustacés Malacostracés, d'un organe comparable à la glande de la mue des Insectes. *C. R. Acad. Sci. Paris*, **237**: 1111–1113.

Gorell, T. A., and L. I. Gilbert, 1969. Stimulation of protein and RNA synthesis in the crayfish hepatopancreas by crustecdysone. *Gen. Comp. Endocrinol.*, **13**: 308–310.

Hinsch, G. W., 1973. Effects of eyestalk ablation on the Y-organ in the spider crab, *Libinia emarginata*. *Amer. Zool.*, **13**: 1353.

Hoffman, D. L., 1969. The development of the androgenic glands of a protandric shrimp. *Biol. Bull.*, **137**: 286–296.

Lochhead, J. H., and M. S. Lochhead, 1941. Studies on the blood and related tissues in *Artemia* (Crustacea, Anostaca). *J. Morphol.*, **68**: 539–632.

Luft, J. H., 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.*, **9**: 409–414.

Madhyastha, M. N., and P. V. Rangneker, 1972. Y-organ of the crab *Varuna litterata* (Fabricius). *Experentia*, **28**: 580–581.

Matsumoto, K., 1962. Experimental studies of the neurosecretory activities of a crab, *Hemigrapsus*. *Gen. Comp. Endocrinol.*, **2**: 4–11.

Novikoff, A. B., 1959. The biochemical cytology of the liver. *Bull. New York Acad. Med.*, **35**: 67–70.

Novikoff, A. B., 1960. Biochemical and staining reactions of cytoplasmic constituents. Pages 167–203 in D. Rudnick, Ed., *Developing Cell Systems and their Control.* Ronald Press, New York.

Passano, L. M., 1960. Molting and its control. Pages 473–536 in T. H. Waterman, Ed., *The Physiology of Crustacea, Volume I.* Academic Press, New York.

Passano, L. M., and S. Jyssum, 1963. The role of the Y-organ in crab proecdysis and limb regeneration. *Comp. Biochem. Physiol.*, **9**: 195–213.

Richardson, K. C., L. Jarett and E. H. Finke, 1960. Embedding in expoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.*, **35**: 313–323.

Scharrer, B., 1966. Ultrastructural study of the regressing prothoracic glands of blattarian insects. *Z. Zellforsch.*, **69**: 1–21.

Scheffé, H. A., 1959. *The Analysis of Variance.* John Wiley and Sons, New York, 477 pp.

Reference: *Biol. Bull.,* **148**: 448–460. (June, 1975)

THE REPRODUCTIVE PHYSIOLOGY OF THE INTERTIDAL PROSOBRANCH *THAIS LAMELLOSA* (GMELIN). II. SEASONAL CHANGES IN BIOCHEMICAL COMPOSITION[1]

WILLIAM B. STICKLE

Department of Zoology and Physiology, Louisiana State University,
Baton Rouge, Louisiana 70803

Relatively few investigations have been published on seasonal changes in the biochemical composition of marine prosobranch gastropods. Blackmore (1969) followed seasonal changes in the level of polysaccharide, lipid, non-protein and protein nitrogen in the limpet *Patella vulgata.* Several papers have been published on seasonal changes in the biochemical level and content of the abalone *Haliotis cracherodii* (Giese, 1969; Webber and Giese, 1968; Webber, 1970). Williams (1970) followed seasonal changes of lipid and carbohydrate level in the periwinkle *Littorina littorea.* Lambert and Dehnel (1974) followed seasonal changes in the biochemical level of several body components and in the digestive gland and gonad indexes of *Thais lamellosa.* They also obtained histological data on feeding activity and gamete maturation.

This investigation deals with seasonal changes in the concentration (level) and absolute quantity (content) of protein, lipid, and polysaccharide in body components of adult *T. lamellosa.* The biochemical composition of its egg capsules and partitioning of female energy during aggregation are also determined. Seasonal changes in the size of the body components (indexes) and respiration rates of this population were previously reported (Stickle, 1973). Aggregation occurred after November 18, 1968 and was completed by March 27, 1969. Egg capsule deposition began between January 7 and 19, 1969 and was completed by March 27, 1969. The accessory reproductive tract of this species consists of an albumin gland and a capsule gland which deposit nourishment for the developing embryos. Lyons and Spight (1973) followed embryological development within the capsule and found snails to emerge from the capsule as crawling juveniles.

METHODS AND MATERIALS

Body components

Animals were collected from Turn Island, Washington and dissected into body components (Stickle, 1973). Male soft body components consisted of the visceral mass and its subdivisions (the testis-digestive gland complex and the remaining visceral mass) and the foot. Female soft body components consisted of the visceral mass and its subdivisions (the ovary-digestive gland complex, the capsule-albumin gland complex and the remaining visceral mass) and the foot.

---

[1] Adapted from portions of a thesis submitted to University of Saskatchewan, Regina Campus in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Samples from the November 1969 and January, February, and March 1970 collections were not analyzed because they thawed during shipment and were not considered suitable for chemical analyses.

Level and content

The level of each biochemical class is presented on a milligrams per gram dry weight basis. Nutrient content is calculated by multiplying the level times the body component index and expressing content in grams. The component index used by Stickle (1973) is the dry weight of the component (× 100) divided by the entire snail weight including the shell and expressed as grams per 100 gram animal.

Chemical analyses

Individual body components were homogenized in diethyl-ether with a Potter-Elvehjem tissue grinder. The homogenate was centrifuged and the ether-lipid solution was poured into a pretared weighing pan. The tissue pellet was washed with another volume of diethyl-ether, centrifuged and the supernatant was added to the same pan. The pan was reweighed after evaporation of the diethyl ether and the weight of the residue was recorded as lipid.

The tissue pellet was rehomogenized in distilled water to a final volume of ten milliliters. Protein and polysaccharide levels were determined on aliquots of this homogenate.

Polysaccharide was isolated from an aliquot of the homogenate by the method of Barnes, Finlayson and Piatigorsky (1963). Polysaccharide level was determined by the Seifter (1950) modification of the anthrone method. Rabbit liver Type III glycogen was used as a standard.

A one milliliter aliquot of the homogenate was precipitated with ten milliliters of 10% trichloroacetic acid (TCA) and centrifuged. The tissue pellet was then washed with five milliliters of 5% TCA, centrifuged, washed with five milliliters of 5% TCA and recentrifuged. Protein nitrogen in the washed precipitate was determined by standard micro-Kjeldahl techniques which included nesslerization. Protein was determined by multiplying protein nitrogen by 6.25.

Statistical analyses

Each parameter was analyzed as the mean plus and minus the confidence interval at the ninety-five percent level of significance.

RESULTS

Visceral mass levels

Male and female visceral mass protein, lipid, and polysaccharide levels are given in Table I. The protein level of both sexes declined sharply during the 1968–69 egg laying period which lasted from January through March. Protein level was higher in December, 1969 than December, 1968. Lipid level decreased from a November prespawning high to the low point in the annual cycle in Feb-

TABLE I

Biochemical levels of visceral mass in mg dry weight^{-1}.

Month	Males						Females					
	Protein	N	Lipid	N	Poly.	N	Protein	N	Lipid	N	Poly.	N
Sept. 68	384 ± 255	12	165 ± 23	13	81 ± 14	9	500 ± 83	9	141 ± 20	11	59 ± 14	10
Oct. 68	432 ± 39	9	98 ± 12	11	92 ± 16	9	434 ± 28	17	97 ± 6	16	99 ± 15	9
Nov. 68	456 ± 53	9	154 ± 20	9	75 ± 11	8	526 ± 50	10	143 ± 24	10	72 ± 9	10
Dec. 68	444 ± 27	9	95 ± 12	12	67 ± 12	12	514 ± 214	12	112 ± 14	12	52 ± 13	12
Jan. 69	450 ± 40	12	64 ± 11	12	58 ± 12	11	554 ± 91	10	93 ± 11	12	51 ± 6	9
Feb. 69	362 ± 31	12	62 ± 15	12	28 ± 7	12	434 ± 74	11	99 ± 21	12	24 ± 9	12
Mar. 69	305 ± 37	12	86 ± 23	12	65 ± 15	8	395 ± 33	12	80 ± 18	12	56 ± 20	8
Apr. 69	362 ± 78	8	48 ± 13	8	39 ± 11	8	482 ± 83	8	60 ± 21	8	37 ± 11	8
May 69	513 ± 110	6	61 ± 7	8	29 ± 10	8	571 ± 72	8	74 ± 13	8	28 ± 8	7
June 69	401 ± 60	12	76 ± 20	12	57 ± 11	10	436 ± 62	11	73 ± 17	12	46 ± 9	10
July 69	475 ± 37	10	67 ± 14	10	61 ± 19	9	474 ± 46	10	67 ± 10	10	53 ± 17	10
Aug. 69	522 ± 40	7	54 ± 12	8	61 ± 8	8	453 ± 100	8	74 ± 17	8	49 ± 8	8
Sept. 69	487 ± 32	8	141 ± 37	8	83 ± 6	7	587 ± 22	8	125 ± 23	8	75 ± 11	8
Dec. 69	613 ± 34	8	88 ± 31	8	91 ± 17	8	627 ± 57	7	126 ± 23	8	73 ± 15	7

ruary. Male lipid level increased in March, which may have been due to resumed feeding activity or the sharp concomitant decrease in protein level. Males tend to leave the breeding aggregation and begin feeding before females. Female visceral mass lipid level continued to decline through April. Lipid level remained low through the summer months for both sexes and increased sharply from August to September, 1969. Polysaccharide level of both sexes declined continuously from

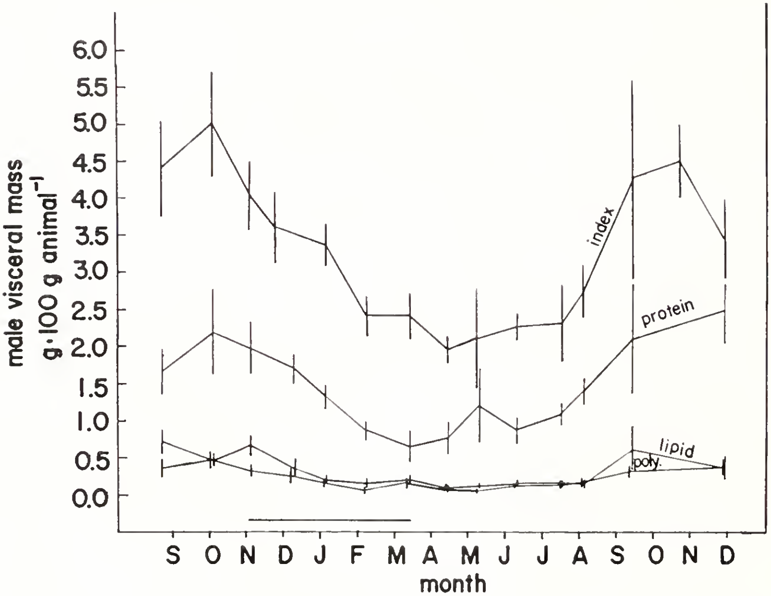


FIGURE 1. Male visceral mass content of the index, protein, lipid and polysaccharide are represented by lines. Vertical lines through each line on the respective sampling dates represent the ninety-five per cent confidence interval about that mean value. The horizontal line at the bottom of the figure indicates the period of male aggregation.

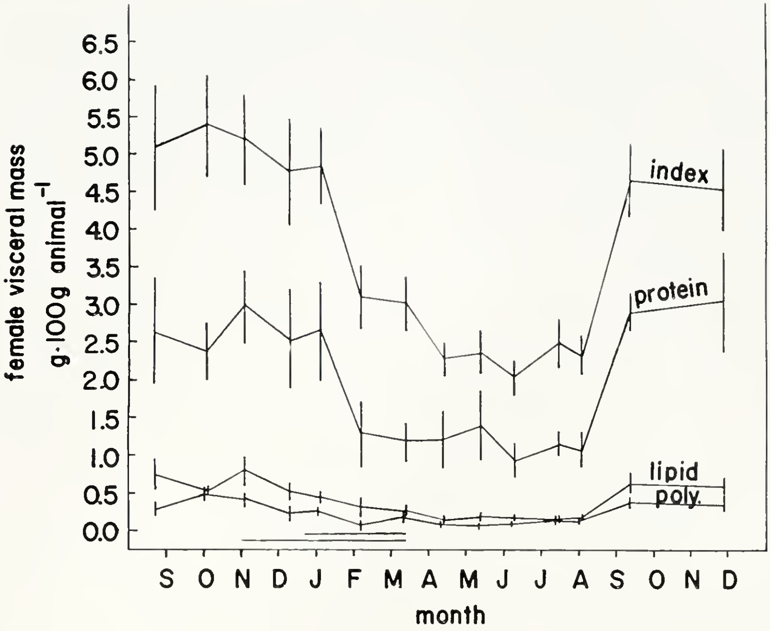


FIGURE 2. Female visceral mass content of the index, protein, lipid and polysaccharide are represented by lines. Vertical lines through each line on the respective sampling dates represent the ninety-five per cent confidence interval about that mean value. The longer horizontal line at the bottom of the figure indicates the period of aggregation and the shorter line represents the period of capsule deposition.

October to February. The sharp increase in polysaccharide level of both sexes during March was caused by a concomitant sharp drop in the protein level. Polysaccharide level reached a second low in May and then climbed consistently until the fall of 1969.

Visceral mass content

Male visceral mass indexes and protein, lipid and polysaccharide contents are given in Figure 1 and female data are given in Figure 2. Protein was the dominant biochemical class and its content closely paralleled seasonal changes in the visceral mass indexes. Lipid content increased just prior to aggregation and spawning and then declined during spawning and the summer months. It increased again during the fall months. Polysaccharide content changed less than protein or lipid but followed the same general pattern as lipid content.

Complex component level and content

Seasonal changes in male testis-digestive gland, remaining visceral mass and foot protein, lipid and polysaccharide level and content are given in Table II. Highest protein levels occurred during the fall in the testis-digestive gland and foot components and the lowest level occurred during the latter part of the aggregation period of February. Protein level in the remaining visceral mass was lowest during September concomitant with a sharp rise in the polysaccharide level. Lipid was much more concentrated in the testis-digestive gland than in the other two components with the maximum level occurring during the fall prior to aggregation and copulation. Polysaccharide level was highest in the remaining visceral mass during the fall and in the foot in September, 1969. The lowest levels in the remaining visceral mass and foot occurred during February and May. The testis-digestive gland polysaccharide level was lower than was found in the other two components throughout the year.

Seasonal changes in component content of protein, lipid, and polysaccharide were predominantly determined by changes in the respective indexes. All three biochemical classes exhibited a distinct seasonal cycle in the testis-digestive gland and remaining visceral mass of being highest in the fall, declining during the winter and spring, remaining stable during the summer and increasing again in the fall. No seasonal cycle of body indexes existed for the foot. The foot was a poor depot for lipid and polysaccharide. Foot protein content was higher in the fall of 1969 than in the same period of 1968.

Seasonal changes in female ovary-digestive gland, capsule-albumin gland, remaining visceral mass and foot biochemical levels and contents are given in Table III. Protein level was highest in the capsule-albumin gland complex. Protein reached the lowest level in the ovary-digestive gland, remaining visceral mass and foot during February but the lowest level was not reached in the capsule-albumin gland until May. This may have been due to the fact that capsule deposition was not completed until the end of March. Protein level was higher in all components in the fall of 1969 than in November, 1968. Lipid was considerably more concentrated in the ovary-digestive gland than in the other components and exhibited a distinct seasonal cycle of being highest before aggregation and lowest during spawning (February) and in May. Polysaccharide was most concentrated in the remaining visceral mass where it exhibited a strong seasonal cycle of being highest in the fall and declining during the winter and spring. Polysaccharide level rose sharply between May and September in the capsule-albumin gland, remaining visceral mass and foot.

Seasonal changes in nutrient content were predominantly due to changes in indexes. Protein content was highest in the ovary-digestive gland where it exhibited a strong seasonal cycle. Capsule-albumin gland protein content increased sharply from September to December while the content of the ovary-digestive gland and remaining visceral mass decreased during the same period. Lipid content was only substantial in the ovary-digestive gland with the other three components being very minor lipid depots. Ovary-digestive gland lipid content exhibited a definite seasonal cycle. Polysaccharide content was higher in the ovary-digestive gland and remaining visceral mass than in the capsule-albumin gland and foot. This was mainly due to differences in the index magnitude. A seasonal cycle was observed in all but the foot.

Biochemical budget for a female snail during capsule deposition

Because female snails do not feed while aggregated it was possible to partition the loss of biomass, protein, lipid and polysaccharide from the visceral mass into

TABLE II

Biochemical composition of male T. lamellosa. *Complex components expressed as level = mg/g dry wt; content = g/100 g animal.*

Component	Month	Index*	Protein			Lipid			Polysaccharide		
			Level	Content	N	Level	Content	N	Level	Content	N
Testis-digestive gland	Nov. 68	2.46	393 ± 45	0.881 ± 0.344	5	233 ± 45	0.584 ± 0.222	6	36 ± 23	0.083 ± 0.063	5
	Feb. 69	0.99	351 ± 74	0.356 ± 0.267	8	142 ± 29	0.145 ± 0.112	8	37 ± 25	0.030 ± 0.022	7
	May 69	0.75	474 ± 75	0.388 ± 0.192	7	93 ± 15	0.068 ± 0.026	8	32 ± 9	0.026 ± 0.015	8
	Sept. 69	2.50	510 ± 52	1.297 ± 0.536	8	219 ± 58	0.558 ± 0.295	8	49 ± 7	0.122 ± 0.044	8
	Dec. 69	1.76	487 ± 73	0.988 ± 0.281	8	151 ± 50	0.322 ± 0.144	8	67 ± 31	0.128 ± 0.040	8
Remaining visceral mass	Nov. 68	1.85	499 ± 93	0.814 ± 0.197	5	38 ± 10	0.067 ± 0.017	6	136 ± 56	0.237 ± 0.100	5
	Feb. 69	1.45	530 ± 70	0.721 ± 0.244	7	25 ± 3	0.035 ± 0.011	8	37 ± 24	0.058 ± 0.048	7
	May 69	1.42	526 ± 125	0.758 ± 0.283	7	34 ± 5	0.047 ± 0.004	8	27 ± 12	0.038 ± 0.018	8
	Sept. 69	1.77	475 ± 44	0.832 ± 0.139	8	33 ± 6	0.058 ± 0.014	8	125 ± 14	0.205 ± 0.041	7
	Dec. 69	1.77	545 ± 58	1.057 ± 0.189	8	22 ± 6	0.043 ± 0.016	8	98 ± 18	0.195 ± 0.059	8
Foot	Nov. 68	0.56	461 ± 63	0.268 ± 0.044	9	36 ± 4	0.021 ± 0.003	9	50 ± 17	0.028 ± 0.010	8
	Feb. 69	0.54	406 ± 57	0.241 ± 0.053	10	27 ± 3	0.015 ± 0.002	12	21 ± 3	0.012 ± 0.003	12
	May 69	0.51	482 ± 81	0.251 ± 0.060	7	31 ± 9	0.016 ± 0.005	8	25 ± 8	0.012 ± 0.004	8
	Sept. 69	0.51	593 ± 101	0.324 ± 0.061	8	24 ± 9	0.013 ± 0.005	8	144 ± 22	0.078 ± 0.013	8
	Dec. 69	0.54	641 ± 93	0.375 ± 0.047	8	17 ± 6	0.010 ± 0.003	6	63 ± 14	0.037 ± 0.009	8

* From Stickle, 1973.

TABLE III

Biochemical composition of female T. lamellosa. *Complex components expressed as level = mg/g dry wt; content = g/100 g animal.*

Component	Month	Index*	Protein			Lipid			Polysaccharide		
			Level	Content	N	Level	Content	N	Level	Content	N
Ovary-digestive gland	Nov. 68	2.91	529 ± 78	1.620 ± 0.531	8	236 ± 43	0.723 ± 0.404	8	37 ± 12	0.121 ± 0.067	8
	Feb. 69	1.11	480 ± 128	0.530 ± 0.220	8	194 ± 62	0.253 ± 0.144	8	25 ± 7	0.028 ± 0.014	8
	May 69	0.81	542 ± 62	0.405 ± 0.114	8	149 ± 33	0.116 ± 0.062	8	27 ± 6	0.021 ± 0.008	8
	Sept. 69	2.47	573 ± 15	1.518 ± 0.205	8	206 ± 36	0.559 ± 0.141	8	39 ± 9	0.106 ± 0.028	8
	Dec. 69	2.05	556 ± 62	1.183 ± 0.263	8	250 ± 51	0.518 ± 0.088	8	48 ± 7	0.102 ± 0.023	8
Capsule-albumin gland	Nov. 68	0.94	601 ± 98	0.596 ± 0.182	8	37 ± 7	0.036 ± 0.010	8	56 ± 18	0.054 ± 0.020	8
	Feb. 69	0.54	687 ± 235	0.450 ± 0.369	8	60 ± 23	0.027 ± 0.008	8	51 ± 24	0.024 ± 0.010	8
	May 69	0.39	552 ± 185	0.222 ± 0.185	8	79 ± 27	0.022 ± 0.006	8	49 ± 14	0.014 ± 0.006	7
	Sept. 69	0.61	768 ± 126	0.441 ± 0.132	8	24 ± 6	0.041 ± 0.010	8	131 ± 45	0.072 ± 0.021	8
	Dec. 69	1.04	841 ± 165	1.021 ± 0.245	7	25 ± 6	0.037 ± 0.008	8	56 ± 13	0.066 ± 0.009	7
Remaining visceral mass	Nov. 68	1.63	484 ± 70	0.823 ± 0.172	8	37 ± 9	0.062 ± 0.014	8	107 ± 19	0.188 ± 0.063	8
	Feb. 69	1.12	450 ± 38	0.516 ± 0.121	7	32 ± 8	0.037 ± 0.010	6	17 ± 8	0.021 ± 0.013	7
	May 69	1.31	580 ± 85	0.768 ± 0.208	8	23 ± 4	0.030 ± 0.009	8	22 ± 10	0.031 ± 0.020	8
	Sept. 69	1.66	557 ± 82	0.952 ± 0.169	8	40 ± 30	0.022 ± 0.005	8	115 ± 24	0.192 ± 0.031	8
	Dec. 69	1.44	577 ± 69	0.865 ± 0.166	8	31 ± 8	0.033 ± 0.009	8	120 ± 29	0.179 ± 0.048	8
Foot	Nov. 68	0.63	501 ± 63	0.334 ± 0.055	9	34 ± 4	0.022 ± 0.002	10	46 ± 12	0.031 ± 0.009	9
	Feb. 69	0.59	402 ± 51	0.241 ± 0.053	10	35 ± 5	0.021 ± 0.003	12	24 ± 6	0.015 ± 0.005	8
	May 69	0.59	581 ± 84	0.386 ± 0.066	8	21 ± 7	0.014 ± 0.005	8	28 ± 7	0.018 ± 0.005	8
	Sept. 69	0.66	589 ± 84	0.365 ± 0.063	8	20 ± 3	0.013 ± 0.002	8	103 ± 32	0.062 ± 0.016	7
	Dec. 69	0.60	622 ± 114	0.372 ± 0.071	8	22 ± 8	0.013 ± 0.003	8	67 ± 18	0.040 ± 0.008	8

* From Stickle, 1973.

Table IV

Biochemical budget for a female snail during aggregation.

Biochem. class	Mg deposited per capsule	Capsules* deposited per female	Mg deposited per female	Mg lost during spawning per female**	Per cent lost as spawn per female	Per cent catabolized per female
Protein	2.3	36.2	83.3	166.1	50	50
Lipid	0.6	36.2	21.7	21.3	102	—
Poly.	0.1	36.2	3.6	9.1	40	60

* From Spight (1972).
** From Stickle (1973).

the production of egg capsules or the catabolic process of respiration. Calculations were based on the average female studied which weighed 11.3208 grams, and was 40.9 mm long (Stickle, 1973). Of the 207 milligrams of biomass lost per female during aggregation 168 milligrams, 81% of the total, were used for the production of egg capsules and 39 milligrams, 19% of the total, were used for female catabolism (Stickle, 1973). Forty-three empty egg capsules were collected from Turn Island after juveniles emerged from them, dried and weighed. The average dry weight was 2.08 ± 0.18 milligrams. Production of the empty capsules accounted for 75.3 milligrams, 20.8 mg per capsule × 36.2 capsules per female, or 36% of the total amount of biomass lost per female. Ninety-two milligrams or 45% of the amount lost per female during aggregation were utilized in the production of embryos and nutritive fluids.

Table IV gives the results of the partitioning of female visceral mass protein, lipid and polysaccharide content. Protein loss accounted for 80% of the biomass lost during aggregation as compared to a loss of 10% due to lipid depletion and 4% due to polysaccharide depletion. These values resulted in a protein:lipid: polysaccharide ultilization ratio of 18.3:2.3:1.0.

The biochemical composition of 78 freshly laid egg capsules with an average dry weight of 4.6 milligrams was: protein—508; lipid—129; polysaccharide—23 and ash—128 milligrams per gram dry weight. The content of each constituent deposited per capsule was the product of the constitutent level and 4.6 milligrams per capsule. The content per capsule was multiplied by 36.2 to give the quantity of each constituent deposited in capsules per female.

Protein utilization by females during aggregation was split evenly between catabolism and production of egg capsules. Slightly more lipid was deposited in egg capsules than was lost from the female visceral mass during aggregation. Forty percent of the polysaccharide lost from the visceral mass was deposited in capsules and 60% was catabolized.

Discussion

Seasonal changes in the biochemical content of all components, except the foot, were predominantly due to changes in indexes and not biochemical level. Many investigators have overlooked this fact when studying seasonal changes in the biochemical composition of molluscs. Giese (1969) has emphasized that molluscs lack discrete nutrient storage depots such as the vertebrate liver, the subdermal and omental adipose tissue of mammals and the fat bodies of lower vertebrates. Therefore nutrient storage occurs primarily through the production of new cellular elements. Seasonal shifts in protein, lipid and polysaccharide levels are merely reflections of their relative rates of synthesis and degradation and are often meaningless with respect to seasonal shifts in their content.

Protein content within the visceral mass was much higher than lipid and polysaccharide content and it exhibited a distinct seasonal cycle. Lambert and Dehnel (1974) also found protein to be the dominant constituent of the gonad and digestive gland in *T. lamellosa.* Giese (1969) reported protein to be the dominant organic constituent in molluscs. The female capsule-albumin gland had the highest protein level but the highest content was found in the ovary-digestive gland. Protein level cycled in all three components of the male. Testis-digestive gland and remaining visceral mass content exhibited a distinct seasonal cycle.

Protein level for comparable tissue was somewhat lower in this study than was reported in Lambert and Dehnel's (1974) work. Part of the difference in the results of the two studies may have been due to methods used. Giese (1966) pointed out that protein determinations based on the Kjeldahl method yield lower values than those obtained from the Lowry method used by Lambert and Dehnel. Population differences also exist. A population of snails from near Juneau, Alaska had much lower protein and polysaccharide levels and higher lipid levels in the visceral mass on all sampling dates than existed in the Turn Island population.

Lipid content was highly concentrated in the gonad-digestive gland complex and its seasonal cycle was mainly due to index changes. Giese (1969) suggests that a lipid value of 5% of dry weight (= 50 mg/g) is a good estimate of structural lipid. Lipid levels exceed 5% in the gonad-digestive gland complex only so that none of the other components acts as a depot area. Lambert and Dehnel (1974) found the lipid level of both the digestive gland and the gonad to be high enough that these components should be considered depots. Transfer of lipid between the two components is unlikely because the level and content of both components followed similar trends throughout the study. Giese (1969) indicates that an inverse relationship between these components provides indirect evidence that nutrient transfer is occurring.

It should be emphasized that the diethyl-ether method used in the present investigation would give lower values than the chloroform-methanol methods utilized by Giese (1969) and Lambert and Dehnel (1974). Structural phospholipids are not soluble in diethyl ether.

Polysaccharide levels and contents were lower than lipid values for most of the study. They were higher in the remaining visceral mass of both sexes than in the other components. Values obtained in this study for the gonad-digestive gland complex agree with those obtained by Lambert and Dehnel (1974) for the gonad and digestive gland analyzed separately. Polysaccharide was likely glycogen because Emerson (1965) found only glucose in the polysaccharide fraction of whole *T. lamellosa.*

Lambert and Dehnel (1974) found glycogen and protein levels of the digestive gland to increase in parallel with the digestive gland index between April and

August. In August the lipid level began to rise, reaching its maximum level in December while glycogen and protein levels fell. Williams (1970) also found changes in total carbohydrate to occur slightly prior to changes in lipid level. Lambert and Dehnel (1974) felt that lipid must have been synthesized from glycogen. They also stated that the correlation between maximum feeding and peak glycogen levels strongly suggests that glycogen is not stored to any great extent but is either metabolized or converted into lipid or protein. The tremendous increase in visceral mass index between August and September, 1969 was explained by Stickle (1973) as being due to the availability of optimal size barnacles for predation by *T. lamellosa.* Polysaccharide level and content increased tremendously between May and September in the male remaining visceral mass and foot. Foot polysaccharide content declined from September to December. A similar large increase in polysaccharide level and content occurred between May and September in the female capsule-albumin gland, remaining visceral mass and foot. Polysaccharide level and content did not change between September and December in any female body component. Lipid content decreased in the male visceral mass (testis-digestive gland and remaining visceral mass). Protein content increased 2.3 fold in the female capsule-albumin gland complex. If seasonal changes in biochemical composition are based on content, polysaccharide does not act as a temporary storage pool from which lipid and protein are synthesized in *T. lamellosa*.

Stickle and Duerr (1970) and Stickle (1971) found *T. lamellosa* to utilize whatever substrate was available at the time of starvation. Lipid was present in female *T. lamellosa* during aggregation. As indicated above, however, it did not appear to be used as a source of energy for the female during aggregation. Might it be destined for use in reproductive activity in a manner similar to the function of galactogen in the pulmonate *Helix pomatia?* May (1932a, b) found galactogen to be present exclusively in the albumin glands and in the eggs, and glycogen exclusively in the rest of the body. When starved *H. pomatia* utilized glycogen reserves for the first ten days before galactogen reserves were drawn upon. Martin (1961) suggested that galactogen acts as a reserve which is not easily drawn upon and enhances the chances of reproductive success.

The foot was a minor depot for protein, lipid and polysaccharide in *T. lamellosa.* Lambert and Dehnel (1974) came to the same conclusion with their study of *T. lamellosa.* Webber (1970) speculated that the foot of *H. cracherodii* may act as a storage depot for metabolic demands of gonad growth. The foot is a predominate body component of *H. cracherodii* but only a minor one in *T. lamellosa* (Stickle, 1973). Giese (1969) stated that the molluscan foot can hardly be considered a lipid storage organ.

Much of the protein deposited in egg capsules was likely used for capsule wall production. Fretter and Graham (1962) summarized the capsule wall composition of *Nucella* (= *Thais*) *lapillus* as being composed of three layers of protein and a little mucoprotein.

Lipid was present in much higher concentration in *T. lamellosa* capsules (12.9%) than has been found in pulmonate and opisthobranch spawn. Bayne (1968) histochemically analyzed the reproductive products of eight gastropods (six pulmonates, one opisthobranch, and one prosobranch). Lipid was present in the nutritive fluid of *Nucella* (= *Thais*) *lapillus* only. Polysaccharide appears to be the nutritive substrate deposited for an energy source of embryonic pulmonate snails (Bayne, 1966; 1968). Stickle (unpublished data) has analyzed the reproductive products of five prosobranch species and five species of opisthobranchs. Lipid levels are much higher in the prosobranch reproductive material (7–17% of dry weight) than in that of opisthobranchs (1–2% of dry weight). This trend holds even when lipid level is placed on a per gram ash free dry weight basis because of the high ash levels in the opisthobranch reproductive material analyzed (35–62% of dry weight). Polysaccharide levels were low for all species of prosobranchs and opisthobranchs studied (1–2% of dry weight) except for the freshwater prosobranch *Viviparus subpurpureus* (9% of dry weight). Protein levels were a reflection of the type of capsular material deposited around the embryos.

Basic metabolic differences exist in the strategies employed by prosobranchs, pulmonates and opisthobranchs to provide nourishment for embryonic development. Phylogenetic, life history and environmental modifications of the biochemical composition of gastropod reproductive products have received little attention by investigators. The advantage of using species which produce encapsulated spawn is that it allows one the opportunity to determine output per female and determine partitioning of biochemical compounds by the female into those going into spawn production and those used for female catabolic activity.

Those gastropods which aggregate and starve while producing egg capsules utilize energy for body maintenance in addition to producing reproductive products. The catabolic expenditure by females during aggregation must be accounted for when determining the efficiency of alternative reproductive strategies. Stickle and Mrozek (1973) found 30% of the prespawning soft body weight of *Fusitriton oregonensis* to be lost during aggregation. Calculations from data presented by Stickle (1973) indicate 34% of the soft body weight of *T. lamellosa* was lost during aggregation.

While there are no data available on the percent of soft body weight lost during spawning by broadcast fertilizing gastropods, data exist for the clam *Tellina tenuis*. Ansell and Trevallion (1967) found the body weight of a standard size clam to drop from 35 to 30 milligrams during spawning which represents a 14% decline in body weight.

Additional information is needed on the percentage of soft parts utilized in the reproductive effort of animals using different reproductive strategies. The present study indicates that the energetic costs of aggregation, starvation and production of encapsulated embryos are just as high as those for the broadcast fertilization reproductive strategy.

This research was partially supported by a National Research Council of Canada research assistantship. I would like to extend my deepest thanks to Dr. Frederick Duerr, my advisor. Special thanks also go to Dr. Robert Fernald, former Director of the University of Washington's Friday Harbor Laboratories for the use of their excellent facilities.

SUMMARY

1. Seasonal changes in protein, lipid and polysaccharide level and content were studied in several body components of *Thais lamellosa*. Male soft body compo-

nents consisted of the visceral mass, including the testis-digestive gland and remaining visceral mass, and the foot. Female soft body components consisted of the visceral mass, including the ovary-digestive gland, capsule-albumin gland, and remaining visceral mass, and the foot.

2. Seasonal changes in protein, lipid and polysaccharide content in body components, except the foot, were predominantly due to changes in indexes and not biochemical level. The foot was not important as a depot for any of the biochemical constituents. Protein was the dominant constituent in the visceral mass, lipid was intermediate in level and content and polysaccharide level and content were lowest.

3. The gonad-digestive gland complex was a lipid depot for both sexes. No other body component of either sex served as a lipid depot.

4. The ovary-digestive gland lipid content was at its maximum value in September but the capsule-albumin gland protein content did not reach its maximum value until December when it was 2.3 times the September value. The accessory reproductive tract reaches its maximum size just prior to capsule deposition.

5. The ratio of protein:lipid:polysaccharide content lost from the female visceral mass during aggregation was 18.3:2.3:1.0. Protein loss was split evenly between female catabolism and egg capsule production. Lipid loss went entirely to egg capsule production. Polysaccharide loss was split between female catabolism and egg capsule production.

6. Individuals interested in the efficiency of alternate reproductive strategies must consider the costs of aggregation and capsule wall deposition to species utilizing this type of strategy. Energetic costs of aggregation, starvation and production of encapsulated embryos are just as high as costs involved in broadcast fertilization among molluscs.

LITERATURE CITED

Ansell, A., and A. Trevallion, 1967. Studies on *Tellina tenuis* Da Costa: I. Seasonal growth and biochemical cycle. *J. Exp. Mar. Biol. and Ecol.*, **1**: 220–235.

Barnes, H., D. Finlayson and J. Piatigorsky, 1963. The effect of desiccation and anaerobic conditions on the behavior, survival, and general metabolism of three common cirripedes. *J. Anim. Ecol.*, **32**: 233–252.

Bayne, C. J., 1966. Observations on the composition of the layers of the egg of *Agriolimax reticulatus*, the gray field slug (Pulmonata, Stylomatophora). *Comp. Biochem. Physiol.*, **19**: 317–338.

Bayne, C. J., 1968. Histochemical studies on the egg capsules of eight gastropod molluscs. *Proc. Malac. Soc. Lond.*, **38**: 199–212.

Blackmore, D. T., 1969. Studies of *Patella vulgata* L. II. Seasonal variation in biochemical composition. *J. Exp. Mar. Biol. Ecol.*, **3**: 231–245.

Emerson, D. N., 1965. Summer polysaccharide content in seven species of west coast intertidal prosobranch snails. *The Veliger*, **8**: 62–66.

Fretter, V., and A. Graham, 1962. *British prosobranch molluscs.* Ray Society, London, 755 pp.

Giese, A. C., 1966. Lipids in the economy of invertebrates. *Physiol. Rev.*, **46**: 244–298.

Giese, A. C., 1969. A new approach to the biochemical composition of the mollusc body. *Oceanogr. Mar. Biol. Annu. Rev.*, **7**: 175–229.

Lambert, P., and P. A. Dehnel, 1974. Seasonal variations in biochemical composition during the reproductive cycle of the intertidal gastropod *Thais lamellosa* Gmelin (Gastropoda, Prosobranchia). *Can. J. Zool.*, **52**: 305–318.

Lyons, A., and T. M. Spight, 1973. Diversity of feeding mechanisms among embryos of Pacific Northwest *Thais*. *The Veliger*, **16**: 189–194.

MARTIN, A. W., 1961. The carbohydrate metabolism of the Mollusca. Pages 35–64 in A. W. Martin, Ed., *Comparative physiology of carbohydrate metabolism in heterothermic animals.* Univ. of Washington Press, Seattle, Washington.

MAY, F., 1932a. Beitrag zur Kenntnis des glykogen und galaktogen—gehaltes bei *Helix pomatia. Z. Biol.,* **92**: 319–324.

MAY, F., 1932b. Über den galaktogengehalt der eier von *Helix pomatia. Z. Biol.,* **92**: 325–330.

SEIFTER, S., 1950. The estimation of glycogen with the anthrone reagent. *Arch. Biochem.,* **191**: 24–25.

SPIGHT, T., 1972. Patterns of change in adjacent populations of an intertidal snail, *Thais lamellosa. Ph.D. thesis, University of Washington,* 311 pp.

STICKLE, W. B., 1971. The metabolic effects of starving *Thais lamellosa* immediately after spawning. *Comp. Biochem. Physiol.,* **40A**: 627–634.

STICKLE, W. B., 1973. The reproductive physiology of the intertidal prosobranch *Thais lamellosa* (Gmelin). I. Seasonal changes in the rate of oxygen consumption and body component indexes. *Biol. Bull.* **144**: 511–524.

STICKLE, W. B., AND F. G. DUERR, 1970. The effects of starvation on the respiration and major nutrient stores of *Thais lamellosa. Comp. Biochem. Physiol.,* **33**: 689–695.

STICKLE, W. B., AND J. P. MROZEK, 1973. Seasonal changes in the body component indices of the subtidal prosobranch *Fusitriton oregonensis. The Veliger,* **16**: 195–199.

WEBBER, H. H., 1970. Changes in the metabolite composition during the reproductive cycle of the abalone *Haliotis cracheroidii* (Gastropoda: Prosobranchiata). *Physiol. Zool.,* **43**: 213–231.

WEBBER, H. H., AND A. C. GIESE, 1969. Reproductive cycle and gametogenesis in the black abalone *Haliotis cracheroidii* (Gastropoda: Prosobranchiata). *Mar. Biol.,* **4**: 152–159.

WILLIAMS, E. E., 1970. Seasonal variations in the biological composition of the edible winkle *Littorina littorea* (L). *Comp. Biochem. Physiol.,* **33**: 655–661.

Reference: *Biol. Bull.*, **148**: 461–471. (June, 1975)

STUDIES ON THE REPRODUCTIVE SYSTEMS OF SEA-STARS. II. THE MORPHOLOGY AND HISTOLOGY OF THE GONODUCT OF *ASTERIAS VULGARIS*[1]

CHARLES WAYNE WALKER

Cornell University, Ithaca, New York, and Shoals Marine Laboratory, Isles of Shoals, Maine

Although they are directly involved in the important process of shedding gametes, the gonoducts of asteroids have been described in structural terms for only a few species. In fact, the question of whether gonoducts exist in some sea-stars was debated for many years (Müller and Troschel, 1842; Greeff, 1872; Hoffman, 1872; and Ludwig and Hamann, 1899). Those investigators who have studied the gonoducts of asteroids consider either the morphology or the histology of only limited regions of the duct. The general orientation of the gonoduct in the ray is outlined by Müller and Troschel (1842) for *Asterias rubens* and *Crossaster papposus*, by M. Sars (1861) for *Pteraster pulvillus* and *P. militaris*, by Cuénot (1887) for *Marthasterias glacialis*, and by Richters (1912) for *Linckia multiforis*. The histology of the gonoduct is described in a preliminary way by Ludwig (1878) for *Asterina pentagona*, by Hamann (1885) for *Asterias rubens*, and by Gemmill (1911, 1912, and 1914) for *Solaster endeca* and *Asterias rubens*. Some details of the nature of the epithelial cells of the gonoduct of *Asterina gibbosa* are revealed in the ultrastructural studies of Truslé (1969). The present work, dealing with *Asterias vulgaris*, gives a detailed description of the morphology and histology of the entire gonoduct; observations are made on the reproductive systems of immature and mature specimens of both sexes. Such information, provided by integrating morphological and histological observations, will serve as a basis for comparison with the gonoducts of other asteroids and is necessary in interpreting the activities of this important part of the reproductive system.

MATERIALS AND METHODS

Procedures for sexually mature specimens

Twenty-five of the sea-stars used in this study are the same specimens obtained in "major collections" and studied in an earlier work concerning the morphology and histology of the wall of the gonad of *Asterias vulgaris;* methods of identifying, measuring, dissecting, fixing, embedding, and sectioning these specimens have already been described in some detail (Walker, 1974). Two interradii from each of these specimens were stained for use in studies of the morphology and histology of the gonoduct. One entire interradius was stained with Mallory's Phosphotungstic Acid Haematoxylin (PTAH) (Lillie, 1965) for observations on the general histology of the gonoduct. Wherever possible, the number of subsidiary gonoducts (see observations) in each unit of the reproductive system was recorded. The 2 gonoducts in another interradius were stained with aldehyde fuchsin (Cameron and Steele, 1959) to indicate the presence of elastic connective tissue in the gonoduct.

---

[1] This paper represents part of a thesis submitted to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Master of Science.

Collection of small sea-stars

In addition to the 25 sexually mature specimens from the "major collections," 54 small sea-stars, with R values (R is the length from the center of the disc along the aboral surface of the ray to the tip) ranging from 8 to 24 mm, were collected from a variety of locations on the windward side of Star Island, New Hampshire. Thirty were identified as *Asterias vulgaris* on the basis of their major pedicellariae, which were of the elongate pointed variety characteristic of this species. These specimens were fixed and decalcified in Bouin's fluid, dehydrated in ethanol, embedded in paraffin in a vacuum oven, and sectioned at 9 to 12 μ. All specimens were routinely stained with PTAH and were used to investigate the development of the gonoduct.

Wax model

To aid in visualizing the complicated structural details of Region A of the gonoduct (see observations), a three-dimensional wax model was constructed for a sexually mature specimen of each sex. The model was based on 40 serial longitudinal sections of the gonoduct that had all been stained with PTAH. A greatly enlarged wax section was cut to conform to the projected image of each histological section (Sack, 1966); the wax sections were then carefully assembled and studied.

OBSERVATIONS

The reproductive system of normal 5-rayed specimens of *Asterias vulgaris* consists of 10 separate units, each including a gonad, genital branches of the aboral haemal and coelomic (perihaemal) rings, and a gonoduct (Fig. 1). Two units are found in a ray, one attached to either side above the supramarginal ossicles. The gonad is a single large bag-like structure that is composed of two distinct groups of tissues, the outer and inner sacs; the genital branches of the aboral haemal and coelomic (perihaemal) rings which extend from the disc to the gonad are continuous with the haemal and genital coelomic (perihaemal) sinuses, respectively, that are important features in the structure of the wall of the gonad (Walker, 1974).

The gonoduct forms rather late in the life of the sea-star. In the specimens of *Asterias vulgaris* examined in this study, no trace of gonoducts was evident in specimens smaller than 14 mm ray-length. When the gonoduct is first apparent in animals ranging from 15–18 mm ray-length, it is represented by a knob of tissue at the upper or aboral end of the gonad (Fig. 2); even in animals of this size all 10 gonoducts do not begin to form at the same time. In this study, the gonoduct was first recognizable as a completed structure in animals with rays measuring 34 mm, where it is a tube with a single external opening. From its orifice within the gonad, the gonoduct passes through the stalk suspending the gonad and is

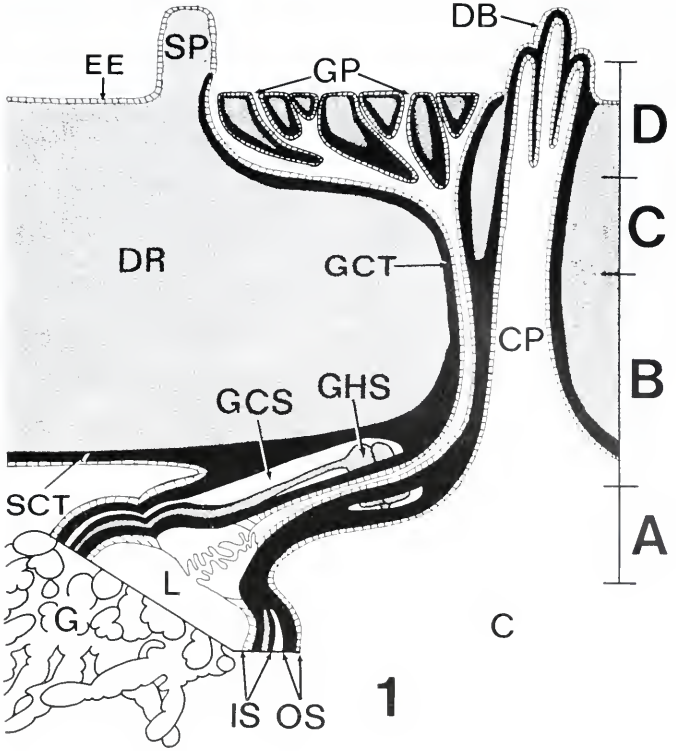
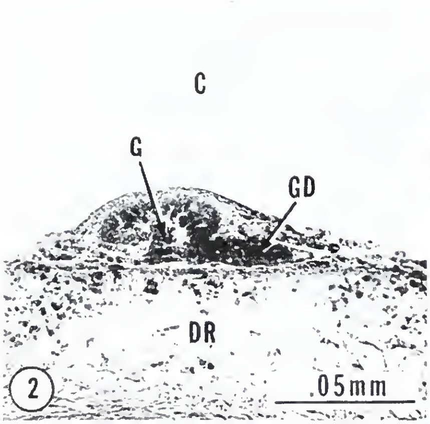
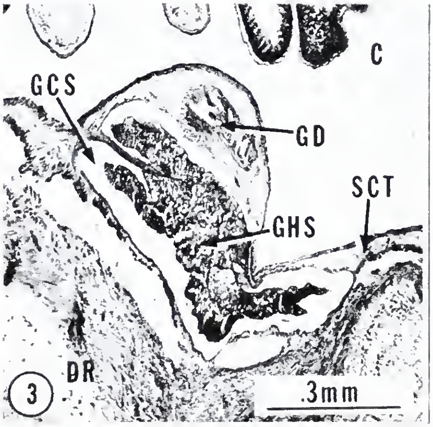
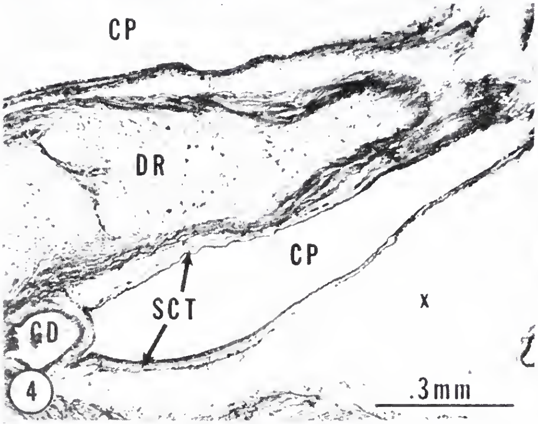


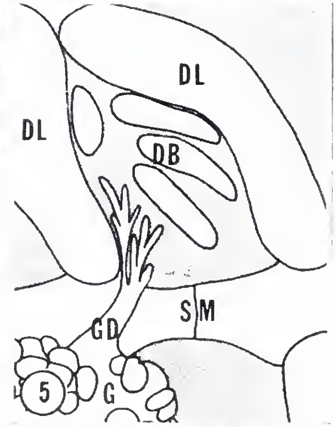
FIGURE 1. Diagrammatic representation of one unit of the reproductive system of *Asterias vulgaris,* showing its major components: the gonad, the gonoduct, and genital branches of the aboral haemal and coelomic (perihaemal) rings. The gonad is shown in external view, while the rest of the unit is shown in longitudinal section. The proportions of various parts of the unit are not representative of their actual dimensions, as several areas are magnified for clarity. Regions A, B, C, and D of the gonoduct are shown in the diagram. Symbols used are: C, coelom; CP; coelomic pocket; DB, dermal branchiae; DR, dermis; EE, external epithelium; G, gonad; GCS, genital coelomic (perihaemal) sinus; GCT, connective tissue of the gonoduct; GHS, genital haemal strand; GP, gonopores; IS, inner sac of the gonad; L, lumen of the gonad; OS, outer sac of the gonad; SCT, subperitoneal connective tissue; SP, spine on a dorsolateral ossicle.

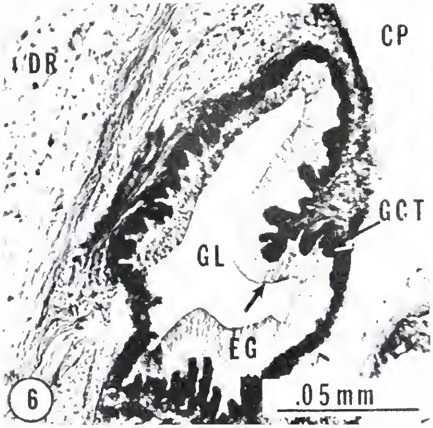
thereafter intimately associated with various tissues in the wall of the ray. As it passes from the gonad to the body wall, the gonoduct accompanies genital branches of the aboral haemal and coelomic (perihaemal) rings, and it follows these proximally and aborally for a variable distance; in section this relationship appears as in Figure 3. The gonoduct soon loses its relationship with the genital branches of the aboral haemal and coelomic (perihaemal) rings and enters a pocket in the body wall that is continuous with the general perivisceral coelom (Figs. 1 and 4, CP); this coelomic pocket leads toward the surface of the ray and subdivides to form the lumina of several dermal branchiae (Fig. 1, DB). It is lined by typical parietal peritoneum underlain by a layer of collagenous connective issue, called the subperitoneal connective tissue layer (Walker, 1974); the gonoduct passes


C
G
GD
DR
2
.05mm


C
GCS
GD
SCT
GHS
DR
3
.3mm


CP
DR
CP
SCT
x
GD
4
.3mm


DL
DL
DB
SM
GD
5
G


DR
CP
GCT
GL
EG
6
.05mm

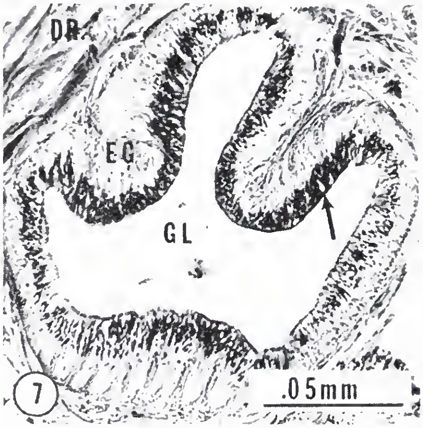

DR
EG
GL
7
.05mm

FIGURE 2. Section of the wall of the ray (R = 18 mm), showing a sexually immature gonad and the bud of the gonoduct (PTAH). Symbols used are: C, coelom; DR, dermis; G, gonad; GD, gonoduct.

FIGURE 3. Section of the wall of the ray near the point of attachment of the gonad, showing the relationships of the gonoduct and genital branches of the aboral haemal and

along one wall of the coelomic pocket within the subperitoneal connective tissue (Figs. 1 and 4, SCT). It is by means of this pocket that the gonoduct is brought near the surface of the ray without actually making its way independently through the entire dermis. In the region where dermal branchiae first branch from the coelomic pocket, the gonoduct leaves the subperitoneal connective tissue of the pocket, penetrates the dermis, and passes the remaining distance to the external epithelium. Within the dermis of young animals, the gonoduct forms a tube with a single lumen that is continuous to the outside; in older animals subsidiary gonoducts form from the primary duct and each passes separately to the surface of the ray. Subsidiary ducts may also be subdivided; one male specimen (R = 56 mm) examined in this study had 17 distinct subsidiary gonoducts associated with one gonad. The number of such ducts in one reproductive unit bears no relationship to that in any other unit in the same sea-star.

Unlike the conspicuous gonopores of *Marthasterias glacialis* (Cuénot, 1887) or those of *Leptasterias groenlandica* (Lieberkind, 1920), which are set on knobs of epithelium, the gonopores of *Asterias vulgaris* are flush with the surface of the ray and are extremely difficult to distinguish with the unaided eye. Only during or just after spawning are they readily observable. They open aborally on the interradial surface of the ray in an interossicular space which is penetrated by several dermal branchiae. The dermal branchiae open internally into a coelomic pocket which is located below the space. The interossicular space is one of hundreds present in the body wall and is bounded orally by supramarginal ossicles and aborally by two to many dorsolateral ossicles (Fig. 5). Gonopores found within the space bounded by these ossicles open in the external epithelium covering either the space or the dorsolateral ossicles themselves.

In terms of its histology, the gonoduct is an extension of the inner sac of the gonad (Fig. 1, IS) (Walker, 1974). The tubular duct is composed of an outer connective tissue layer that is continuous with the inner and outer walls of the haemal sinus of the gonad and also an inner epithelial layer which is continuous with the germinal epithelium of the gonad. No genital haemal strand lies within the connective tissue of the gonoduct and neither muscle nor nerve fibers are recognizable among the epithelial cells lining the gonoduct.

---

coelomic (perihaemal) rings (PTAH). Symbols used are: C, coelom; DR, dermis; GCS, genital coelomic (perihaemal) sinus; GD, gonoduct; GHS, genital haemal strand; SCT, subperitoneal connective tissue layer.

FIGURE 4. A section of the wall of the ray, showing the relationship of the gonoduct and a coelomic pocket (PTAH). The × marks a large region formed as tissues have lost their normal connection to the dermis. Symbols used are: CP, coelomic pocket; DR, dermis; GD, gonoduct; SCT, subperitoneal connective tissue layer.

FIGURE 5. Diagrammatic representation of the inner lateral wall of the ray showing an interossicular space and also the relationship of the gonad and the gonoduct to the ossicles of the ray. Symbols used are: DB, dermal branchiae; DL, dorsolateral ossicles; G, gonad; GD, gonoduct; SM, supramarginal ossicles.

FIGURE 6. Cross-section of Region B of the gonoduct; notice the dark staining folded connective tissue layer (Aldehyde fuchsin). The arrow indicates a vacuolated cell. Symbols used are: CP, coelomic pocket; DR, dermis; EG, epithelium of the gonoduct; GCT, connective tissue of the gonoduct; GL, lumen of the gonoduct.

FIGURE 7. Cross-section of Region C of the gonoduct, showing the typical disposition of the epithelial and connective tissue layers (PTAH). The arrow indicates a vacuolated cell. Symbols used are: DR, dermis; EG, epithelium of the gonoduct; GL, lumen of the gonoduct.

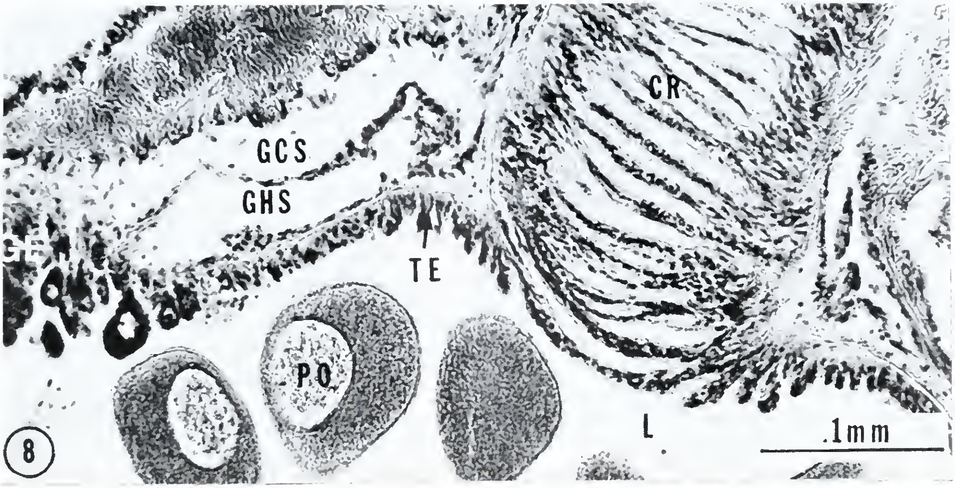
The outer connective tissue layer is characteristically thrown into ridges which run longitudinally along the length of the gonoduct; the fibers of this layer also run longitudinally along the axis of the duct, and they stain darkly with aldehyde fuchsin (Fig. 6). Toward the lumen of the gonoduct, these ridges of connective tissue support the epithelium of the gonoduct which is composed of common flagellated and scattered vacuolated cells (Figs. 6 and 7, EG and arrows). The flagellated cells form a pseudostratified epithelium of crowded columnar cells, often with extremely attenuated bases. Nuclei are usually ovoid with their long axes oriented perpendicularly to the basement membrane. At its free end, each cell bears a single flagellum that rises from a distinct basal body. In favorable preparations, a clear brush border of microvilli is evident. The vacuolated cells are found along the length of the gonoduct of either sex among the much more common flagellated cells. Typically, each contains a large ovoid vacuole, with maximum dimensions of 5 $\mu \times 12$ μ, which may be located at any height in the layer of cells. Often these vacuoles appear to open into the lumen of the gonoduct.

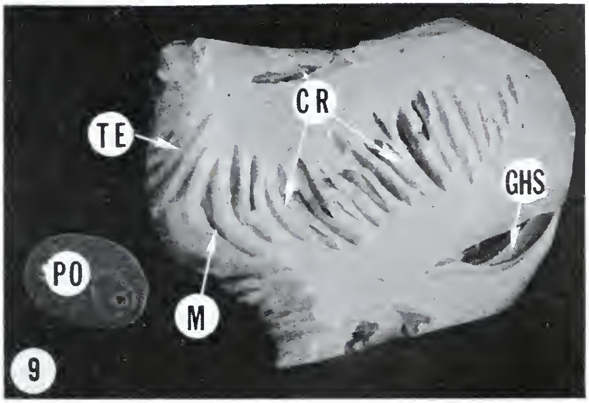
To facilitate further description, the gonoduct has arbitrarily been divided into four regions along its length (Fig. 1; A, B, C, and D).

Region A: although this is the shortest portion of the gonoduct it is at the same time the most complex in terms of the relationships of its connective tissue and in terms of the complexity of its epithelial layer. This is an area of transition between the tissues of the inner sac of the gonad and those of the gonoduct. It is in this region that the gonoduct first forms an association with the subperitoneal connective tissue layer. The stalk suspending the gonad is a cylinder of connective tissue which is continuous between the connective tissue of the outer sac of the gonad and the subperitoneal connective tissue of the body wall. As the gonoduct passes through this cylinder, its connective tissue fibers become firmly knitted on one side to those of the stalk; later, in Region B, the gonoduct is completely surrounded by the subperitoneal connective tissue layer (Fig. 1).

The epithelium lining the lumen of the gonoduct in Region A is extremely complex and only a preliminary account of its nature is given here. A short zone of transitional epithelium lies between the epithelium of the gonoduct and the germinal epithelium (Fig. 8, TE). The following observations, made on the epithelium of the gonoduct, are based both on wax models and on serial sections of Region A. The opening of the gonoduct into the lumen of the gonad is inconspicuous; it is formed by several slit-like openings which are surrounded by a group of concentric circular ridges of transitional epithelium. Within the gonoduct, a series of imbricating ridges of epithelium project into the lumen of the duct (Figs. 8 and 9). The lumen of the duct meanders among these ridges and in its circuitous course gives off blind sacs. There follows a series of ridges formed of epithelium that spiral along the axis of the gonoduct and that eventually fade away leaving the simple unobstructed lumen of Region B. The arrangement of the epithelial layer of the gonoduct in Region A, described above, is obvious in all mature specimens examined. It is not apparent at all in very young animals; in slightly older but still immature specimens it is represented by a region of thick epithelium that occludes the lumen of the gonoduct.

Region B: in contrast to Region A, this is the longest section of the gonoduct and also the simplest in terms of its relationships to the ray and of the complexity





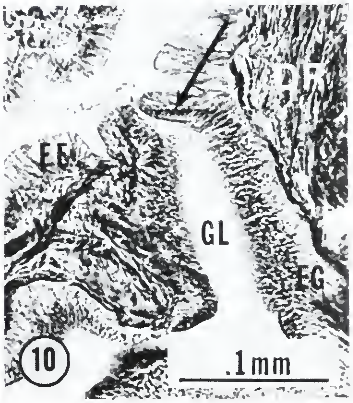


FIGURE 8. A longitudinal section of the gonoduct where it opens into the lumen of the gonad, showing the ridges of epithelium of Region A of the gonoduct, CR (PTAH). Notice the continuity between the germinal epithelium of the gonad and the epithelium of the gonoduct with transitional epithelium intervening. Symbols used are: GCS, genital coelomic (perihaemal) sinus; GE, germinal epithelium of the gonad; GHS, genital haemal strand; L, lumen of the gonad; PO, primary oocyte; TE, transitional epithelium of the gonoduct.

FIGURE 9. Photograph of a wax model reconstructed from serial sections of the gonoduct in Region A (female). Notice the primary oocyte which has a diameter of about 150 μ. Symbols used are: CR, cross-ridges of epithelium in Region A of the gonoduct; GHS, genital haemal strand; M, mouth of the gonoduct; PO, primary oocyte; TE, transitional epithelium.

FIGURE 10. Longitudinal section of the terminal portion of the gonoduct in Region D, showing a subsidiary gonoduct opening at the surface of the ray (PTAH). Notice the distinct change in the epithelia just inside the mouth of the gonoduct (arrow); the opening to external environment is lined by the external epithelium and not by the epithelium of the gonoduct. Symbols used are: DR, dermis; EE, external epithelium; EG, epithelium of the gonoduct; GL, lumen of the gonoduct.

of its tissues (Figs. 1 and 6). Throughout this region, the gonoduct is enclosed by the subperitoneal connective tissue of the body wall. Here, as in Region A, the connective tissue fibers of the gonoduct run longitudinally parallel to the lumen, and the layer that they constitute is thrown into longitudinal folds. These folds are irregularly rounded, some being very short and others long and slender, rising into the epithelial layer (Fig. 6). In the epithelial layer, flagellated cells ride the crests and troughs of the underlying connective tissue, but their free ends all rise to a common level giving a fairly uniform surface to the lumen. To accomplish this, some of the cells are much taller than others; the bases of the tallest cells are very slender, often appearing as mere fibers.

Regions C and D: after proceeding for a variable distance along the coelomic pocket within the subperitoneal connective tissue layer, the gonoduct finally penetrates the wall of the ray. Upon doing so its connective tissue is bound in a new relationship with the dermis. All tissue layers of the gonoduct are similar in Regions C and D and with few exceptions are identical to those seen in Region B. In Region C, the gonoduct often appears roughly circular in cross-section, but usually flattens into a narrow tube near Region D where it is divided into subsidiary gonoducts. The subsidiary ducts have variable diameters, and it is usually impossible to determine which represents the primary gonoduct. There may be one or two main ducts, or all may be large, or all diminutive. Characteristically, the diameter of the lumen decreases as the ducts approach the surface of the ray. Gonoducts of very small diameter may appear to lack a distinct connective tissue layer, and these have an epithelium composed of a single layer of columnar cells. The gonopores are difficult to detect because the external epithelium is pursed over their openings.

DISCUSSION

In spite of their small size, the gonoducts of *Asterias vulgaris* are structurally complex; some striking similarities and significant differences in morphology and histology can be brought out by comparing the gonoduct in *Asterias vulgaris* with those in other sea-stars that have been described by previous investigators.

As mentioned earlier, each gonoduct of a sexually mature specimen of either sex of *Asterias vulgaris* extends from the gonad to the body wall as a single tube and then becomes subdivided to form subsidary ducts that open separately in the external epithelium. This arrangement of the gonoduct was noticed in *Asterias rubens* by Müller and Troschel (1842) and is presumably found in other members of the Asteriidae; it may be characteristic of sea-stars in other families as well, although such information is not available. In other asteroids, the gonoducts are arranged quite differently. In *Solaster endeca,* for example, many ducts are present, and each arises from a separate tubule of the tuft-like gonad of this organism. These ducts pass to the body wall where they may fuse to form only one or a few main ducts that open in the external epithelium (Gemmill, 1911, 1912). In *Crossaster papposus*, separate gonoducts extend from each tubule of the tuft-like gonad to the body wall, but these do not fuse and consequently many gonoducts pass through the body wall and open in the external epithelium (Gemmill, 1911). It is unfortunate that so little information is available concerning the arrangement of the gonoduct in other sea-stars; such information might be useful in correlating the form of the gonoduct with its function in sea-stars with different methods of reproduction.

The association of the gonoduct with a coelomic pocket found in the wall of the ray occurs in many sea-stars. In *Asterias vulgaris* (Forcipulata, Asteriidae), as mentioned, the coelomic pocket is one of hundreds of similar structures opening into the coelom and extending through the body wall to form dermal branchiae. The gonoduct profits from its relationship with the subperitoneal connective tissue layer of the pocket by being conducted nearer the surface of the ray to the point where it opens in the external epithelium. Ludwig (1877) provides an illustration of such a relationship in *Echinaster fallax* (Spinulosa, Echinasteridae), although he does not mention it in his text. Cuénot (1887) noticed this relationship in several species of asteroids and believed that it was of considerable significance. He illustrates this association as it appears in *Marthasterias glacialis* (Forcipulata, Asteriidae) and mentions its occurrence in *Crossaster papposus* (Spinulosa, Solasteridae) and *Asterias rubens* (Forcipulata, Asteriidae). Richters (1912), in his study of *Linckia multiforis* (Phanerozonia, Linckiidae), gives an excellent figure accompanied by a text description indicating that in the serial gonads of this species, the gonoduct extending from each gonadal tuft is associated with a coelomic pocket. It is thus apparent that the relationship between the gonoduct and a coelomic pocket is similar in many distantly-related species of asteroids; in many sea-stars, however, such a relationship has not been reported.

In developmental terms, there is sound rationale for close association of the gonoduct with a coelomic pocket in the wall of the ray. In *Asterias vulgaris*, by the time the gonoduct begins to form, the dermis is already quite thick and tough. Presumably, it is much easier for the growing gonoduct to burrow along the wall of the ray in the subperitoneal connective tissue following this layer into a coelomic pocket, than to penetrate directly through the entire dermis of the ray. Near the dermal branchiae the connective tissue layer becomes thin, and only there does the gonoduct enter the dermis. Because of the widespread occurrence of this association between the gonoduct and a coelomic pocket in asteroids, it is likely that the gonoduct may develop similarly in sea-stars where it is arranged as it is in *Asterias*. Obviously, where such a relationship is absent the developing gonoduct must reach the surface of the ray in some other way.

A relationship between the gonoduct and a coelomic pocket in the wall of the ray is certainly not without functional significance in the adult sea-star. In *Asterias vulgaris* the folded elastic-like connective tissue and the crowded columnar epithelium of the gonoduct are capable of extreme expansion. Rapid release of gametes is facilitated by this expansion and would be impossible if the gonoduct passed through the entire body wall bound tightly in connective tissue on all sides. Considerable space is provided for such expansion since the gonoduct with its encasing subperitoneal connective tissue is anchored firmly to the dermis on one side only and extends freely into the coelomic pocket elsewhere.

The epithelial ridges found near the orifice of the gonoduct within the gonad (Region A) are another feature of the structure of the gonoduct of *Asterias vulgaris* that was recognizable in both sexes of all mature specimens observed. These ridges have never been mentioned in the literature on asteroids; in fact, Gemmill (1914) makes a point of stating that no barriers are present which would prevent spawning in females of *Asterias rubens*. Only Richters (1912) indicates such ridges in a figure of a sectioned gonad and gonoduct of *Linckia multiforis*, but he does not discuss them in his text. The purpose of these structures is problematical, although it is conceivable that they function to hold gametes in the gonad during the expansion that accompanies gametogenesis. The pressure exerted by the increase in size and numbers of gametes during gametogenesis is great, and the plugging mechanism provided by such ridges could be important in preventing leakage of mature or maturing gametes.

Further detailed studies on the structure of the gonoducts of other species of asteroids would provide information necessary in comparing the form and function of this important part of the reproductive system in several sea-stars with different methods of reproduction and would give the basis for a better understanding of the way in which gametes are released from gonad.

I thank Dr. John M. Anderson for his advice and guidance through the course of this study and for his careful review of the manuscript. I also thank Dr. W. Sack of Cornell University School of Veterinary Science for the use of his wax modeling equipment. Considerable assistance, advice, and encouragement were also provided by my wife, Wilise.

SUMMARY

In *Asterias vulgaris* there are 10 separate units of the reproductive system, each with its own distinct gonoduct. The gonoduct forms late in the life of the sea-star as an outgrowth of the inner sac of the gonad. During its formation, each gonoduct grows from the gonad to the body wall where it burrows through a layer of connective tissue located below the parietal peritoneum. The gonoduct follows this subperitoneal connective tissue into a coelomic pocket (which ends externally in several dermal branchiae) and ultimately penetrates the dermis, where it divides into subsidiary ducts which open separately in the external epithelium of the interradius. The gonoduct consists of an outer elastic connective tissue layer continuous with both walls of the haemal sinus of the inner sac of the gonad and an inner epithelial layer continuous with the germinal epithelium of the gonad. Near the point where the gonoduct opens into the lumen of the gonad, the epithelial layer of the gonoduct forms cross-ridges which span the lumen of the duct and imbricate, partially blocking the lumen.

LITERATURE CITED

BRUSLÉ, J., 1969. Aspects ultrastructuraux de l'innervation des gonades chez l'étoile de mer *Asterina gibbosa* P. *Z. Zellforsch. Mikrosk. Anat.*, **98**: 88–98.

CAMERON, M. L., AND J. E. STEELE, 1959. Simplified aldehyde-fuchsin staining of neurosecretory cells. *Stain Technol.*, **34**: 256–266.

CUÉNOT, L., 1887. Contribution à l'étude anatomique des Astérides. *Arch. Zool. Exp. Gén., Ser. 2,* **2**: 1–144.

GEMMILL, J. F., 1911. Adult anatomy of *Solaster endeca*. *Proc. Roy. Phys. Soc. Edinburgh,* **18**: 174–191.

GEMMILL, J. F., 1912. Development of *Solaster endeca*. *Trans. Zool. Soc. London,* **20**: 1–71.

GEMMILL, J. F., 1914. The development and certain points in the adult structure of *Asterias rubens* L. *Phil. Trans. Roy. Soc. London*, **205**: 213–294.

GREEFF, R., 1872. Ueber den Bau der Echinodermen. III. Mitth. *Sitsungsber, Ges. Beförd. Gesamten. Naturwiss. Marburg,* **10**: 158–172.

HAMANN, O., 1885. *Beiträge zur Histologie der Echinodermen, Heft 2. Die Asteriden anatomisch und histologisch untersucht.* Jena, Fisher, 126 pp.

HOFFMANN, C. K., 1872. Zur Anatomie der Asteriden. *Niederland. Archiv. Zool.*, **2**: 1–32.

LIEBERKIND, I., 1920. On a starfish (*Asterias groenlandica*) which hatches its young in its stomach. *Vidensk. Medd. Dan. Naturhist. Foren., Ser. 2,* **72**: 121–126.

LILLIE, R. D., 1965. *Histopathologic technic and practical histochemistry,* [3rd. ed.] New York, McGraw-Hill, 715 pp.

LUDWIG, H., 1877. Beiträge zur Anatomie der Asteriden. *Z. Wiss. Zool.,* **30**: 99–162.

LUDWIG, H., 1878. Über die Genitalorgane der *Asterina gibbosa. Z. Wiss. Zool.*, **31**: 395–400.

LUDWIG, H., AND O. HAMANN, 1899. Echinodermen, II Buch. Die Seesterne. Pages 591–604 in H. G. Bronn, Ed., *Klassen und Ordnungen des Tierreichs*, Bd. 2 Abt. 3. Leipsig, Winter.

MÜLLER, J., AND F. H. TROSCHEL, 1842. *System der Asteriden.* Braunschweig, F. Viewig und Sohn, 134 pp.

RICHTERS, C., 1912. Zur Kenntnis der Regenerationsvorgänge bei *Linckia. Z. Wiss. Zool.,* **100**: 116–175.

SACK, W. O., 1966. Rapid wax modeling. *Anat. Rec.*, **154**: 233–242.

SARS, M., 1861. *Oversigt af Norges Echinodermer.* Christiania, Videnskabs-selskabet, Brøgger and Christie, 166 pp.

WALKER, C. W., 1974. Studies on the reproductive systems of sea-stars. I. The morphology and histology of the gonad of *Asterias vulgaris. Biol. Bull.,* **146**: 661–677.

Reference: *Biol. Bull.*, **148**: 472–482. (June, 1975)

SHELL GROWTH IN THE SCALLOP *ARGOPECTEN IRRADIANS*. I. ISOTOPE INCORPORATION WITH REFERENCE TO DIURNAL GROWTH

ALFRED P. WHEELER, PATRICIA L. BLACKWELDER,[1] AND KARL M. WILBUR

Department of Zoology, Duke University, Durham, North Carolina 27706

Many molluscs have markings in their shells indicative of periodic incremental growth, and in some species the markings are known to be formed daily (see Rhoads and Pannella, 1970; Wilbur, 1972). Periodic shell growth presumes accompanying physiological and biochemical changes relating to protein synthesis, secretion, and calcium transport since these processes are a part of shell formation. Such periodic changes have not been characterized. However, rhythmic changes in the general metabolism of molluscs have been reported (Sandeen, Stephens and Brown, 1954; Brown, Bennett, Webb and Ralph, 1956; Wright, 1971). Environmental factors which may be involved in periodic growth patterns such as photoperiod (House and Farrow, 1968; Wrenn, 1972) would be expected to trigger biochemical events or to change reaction rates.

The incremental nature of growth is strikingly clear in the shells of scallops in which there is a daily pattern of horizontal shell extension and ridge formation (Clark, 1968; this paper, Fig. 4). The horizontal portion is deposited by the mantle when it is extended horizontally, and the ridge is produced by deposition during a period when the mantle edge is curved upward (Wrenn, 1972; Clark, 1974). Because of the diurnal nature of shell deposition in *Argopecten irradians* and the clear differences in the shell pattern within each daily cycle, this bivalve provides excellent opportunities for the investigation of physiological aspects of shell formation.

The objective of the present study is the measurement of calcium carbonate deposition during periodic daily growth of the scallop *A. irradians* employing ^{45}Ca and ^{14}C-bicarbonate. However, we first examined the adequacy of the isotope method in providing a reliable measure of mineralization during short periods. Attention has been given to (1) the rate of attainment of a steady state between radioisotopes in the medium and the shell-forming mantle tissue, (2) isotope incorporation into shell as a linear function of time, (3) exchange between shell and medium, (4) correlation between size of animals and rates of isotope incorporation into shell, and (5) deposition rates in various shell regions. The results have permitted us to define conditions under which isotopes can be employed to measure shell growth and to investigate diurnal variations in mineral deposition.

MATERIALS AND METHODS

Maintenance of animals

Specimens of *A. irradians* were collected by hand during the summer in the Beaufort, North Carolina region. The animals were maintained in tanks with running sea water at 23–25° C. Natural illumination was supplemented with fluorescent lighting during the day. Under these conditions, the scallops grew and formed daily ridges. Some animals were transported to Durham, North Carolina where they were kept in recirculating sea water at 16–18° C under constant fluorescent light. They were fed by constant flow from a mixed culture of diatoms and algae. Periodic examination of the digestive tract of sacrificed individuals indicated that the animals were feeding. These animals did not increase in linear dimensions, nor did they form daily ridges.

---

[1] Present address: Electron Microscope Laboratory, University of South Carolina, Columbia, South Carolina 29208.

Exposure of animals to radioisotopes

For the radioisotope studies, three animals were maintained in one liter of aerated sea water in a large fingerbowl at 23–25° C for approximately 1 hour. Animals displaying swimming behavior were replaced by other animals. Ten to 50 μCi $NaH^{14}CO_3$ (698 μCi/mg, Amersham Searle) and/or $^{45}CaCl_2$ (13.0 μCi/mg, New England Nuclear) in 0.10 ml were then added to the sea water containing the animals. At the termination of exposure to the radioisotopes, the shells were removed and cleaned of tissue, rinsed thoroughly with tap water, dried overnight at room temperature, and weighed. Empty shells were exposed to isotopes under the same conditions as for the living animals.

In light-dark experiments, animals were placed in one l aerated sea water approximately 1.3 meters from two 40-watt fluorescent lamps covered by a frosted plastic sheet. Other animals were placed in one liter of aerated sea water in containers covered to exclude light.

Radioisotope measurements of shell

^{14}C-carbonate incorporated into the shell was measured by cutting pieces from the right valves with a high speed drill fitted with a saw bit. With the exception of the studies on incorporation rates in various shell regions (see Fig. 2), the pieces examined were cut from the ventral edge. They weighed 10 mg to 30 mg and were approximately 5 mm in the dimension perpendicular to the shell edge and included 3–5 mm of the shell edge. Pieces cut from other regions also weighed 10 mg to 30 mg but the dimensions varied depending on the region studied. The pieces were placed in 10-ml flasks with rubber serum stoppers from which plastic center wells (Kontes Glass) were suspended (Speeg and Campbell, 1968). The center wells were filled with 0.4 ml of 1N hydroxide of hyamine in methanol (Packard), and the stopper was carefully inserted in the flask. Two ml 5% trichloroacetic acid (TCA) were injected through serum stopper to dissolve the calcium carbonate of the shell and liberate CO_2 which was then trapped by the hydroxide of hyamine. It was calculated that 0.4 ml of 1N hydroxide of hyamine was sufficient to absorb all the CO_2 liberated from the shell samples. After allowing about one hour for the absorption of CO_2 by the hydroxide of hyamine, the center wells were placed in 10 ml scintillation fluid containing 4.0 g PPO and 50 mgs POPOP per liter of toulene. Samples were counted in a liquid scintillation counter after storage in the dark to reduce fluorescence due to the hyamine solution. Isotope measurements on 3 pieces of each shell were averaged.

If the animals had been incubated in both ^{45}Ca and ^{14}C-bicarbonate, the ^{45}Ca was recovered by drying the dissolved shell residue in the bottom of the flasks at 70° C, redissolving the residue in 2 ml 100% ethanol, and adding the ethanol to 10 ml scintillation fluid. Small variations in the amount of ethanol or variations of calcium salt of the magnitude present in the samples did not affect counting efficiency. The molar amount of calcium and carbonate incorporated into shell was calculated according to Wilbur and Jodrey (1952) assuming a concentration in sea water of 2.5 mM bicarbonate and 9.5 mM calcium.

The proteinaceous component remaining after dissolution of the shell mineral was dissolved by warming in 0.5 ml hydroxide of hyamine. The hydroxide of hyamine was then added to 10 ml scintillation fluid and counted. The protein had negligible radioactivity and was not considered a source of error in interpreting the activity present in the mineral constituents.

The sea water medium was sampled throughout the course of experiments using the same methods for detection of the isotopes as used for shell. The specific activity did not vary by more than 10% during the course of a 5-hour experiment.

Radioisotope measurements of mantle

Following exposure of animals to the radioisotopes, the right mantle was dissected, rinsed in tap water, and dried to constant weight at 70° C. The mantle was then homogenized for two minutes in a motor-driven ground glass mortar and pestle with 2 ml 10% TCA to liberate remaining ^{14}C-bicarbonate and to solubilize the ^{45}Ca. The volume was brought to 10 ml with distilled water and the material centrifuged. The pellet was washed once with 5 ml distilled water. One ml of both supernatants was added to 10 ml Bray's solution (Bray, 1960) and the ^{45}Ca counted.

The TCA-precipitated pellet from the mantle was dried and prepared for counting as described for shell protein. Less than 5% of the extracted activity was associated with the protein after five hours incubation, indicating that essentially all the ^{45}Ca was dissolved and little ^{14}C-bicarbonate remained in the acidified pellet as bicarbonate or was fixed into protein.

Weight of shell protein

In determining protein content of shell, pieces of shell were weighed and dissolved in 5% TCA. The protein was centrifuged and the pellet was washed once in 5% TCA, once in 95% ethanol, and dried to constant weight at 70° C. For measurements of the protein content of the daily shell ridges, the ridges were scraped from the shell with care to avoid damage to the shell surface proper. They were then treated in the same manner as the other pieces of shell.

Scanning electron microscopy

Pieces of shell taken from the edge were coated with gold-palladium, mounted on aluminum stubs coated with silver paint, and examined by scanning electron microscopy.

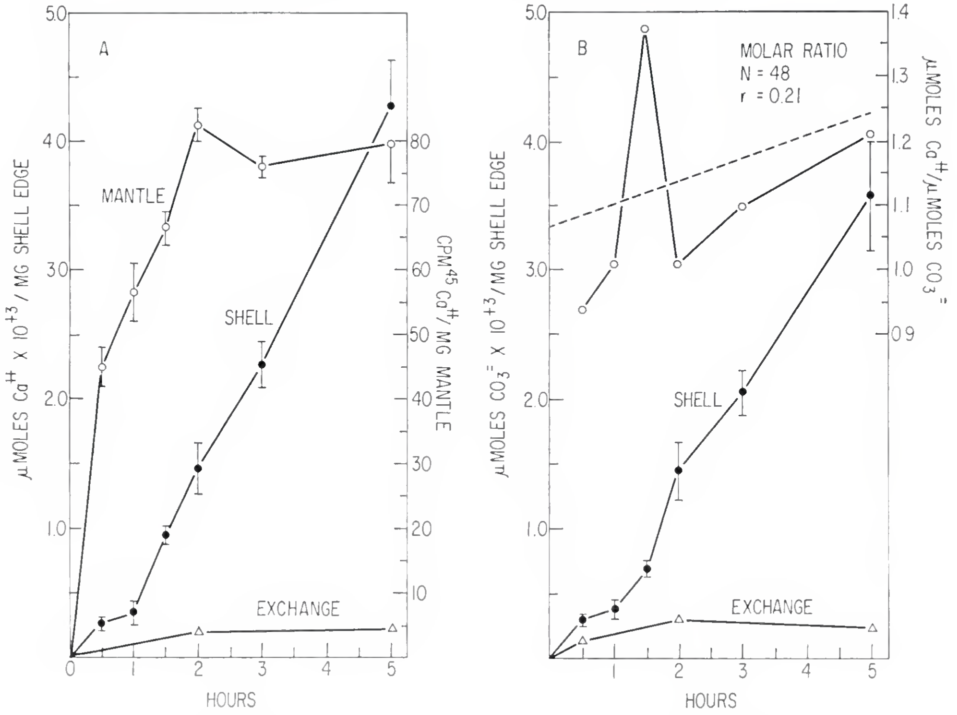


FIGURE 1. (A) Rate of ^{45}Ca incorporation into the right valve, ventral edge (closed circles); and the rate of ^{45}Ca saturation by the right half of the mantle (open circles). Each point represents the mean incorporation for eight animals. The vertical limits indicate standard deviations of the mean. For rate of shell incorporation, triplicate pieces were cut from the ventral edge of each valve, counted, and the results averaged. Exchange of calcium in empty shells is also shown (triangles). The medium contained 6.06×10^4 cpm/ml ^{45}Ca. (B) Rate of carbonate incorporation into the right valve (closed circles); the mean molar ratio of calcium:carbonate at the time intervals indicated (open circles); and the regression line for the molar ratio as a function of time calculated from the individual data points (dotted line). The carbonate incorporation values for each animal were obtained from the same triplicate pieces cut from the shells of the same eight animals as for calcium incorporation (Fig. 1A). The vertical limits indicate standard deviations of the mean. Exchange of carbonate in empty shells is also shown (triangles). The medium contained 2.17×10^4 cpm/ml ^{14}C-bicarbonate.

RESULTS

Uptake of isotopes by mantle and shell

Figure 1A shows the rate of uptake of ^{45}Ca in the mantle and ventral shell edge of animals maintained in running sea water. Uptake of ^{45}Ca in the mantle was rapid and a steady state with the medium was reached in two hours. Deposition of ^{45}Ca in the shell was linear after a lag of about 1 hour. Deposition of ^{14}C-carbonate in shell followed a course similar to that for ^{45}Ca (Fig. 1B). The lag in the case of ^{14}C-carbonate was about 2 hrs. For both isotopes, the exchange with empty shells was 5% to 8% of the total incorporation at five hours (Figs. 1A, B).

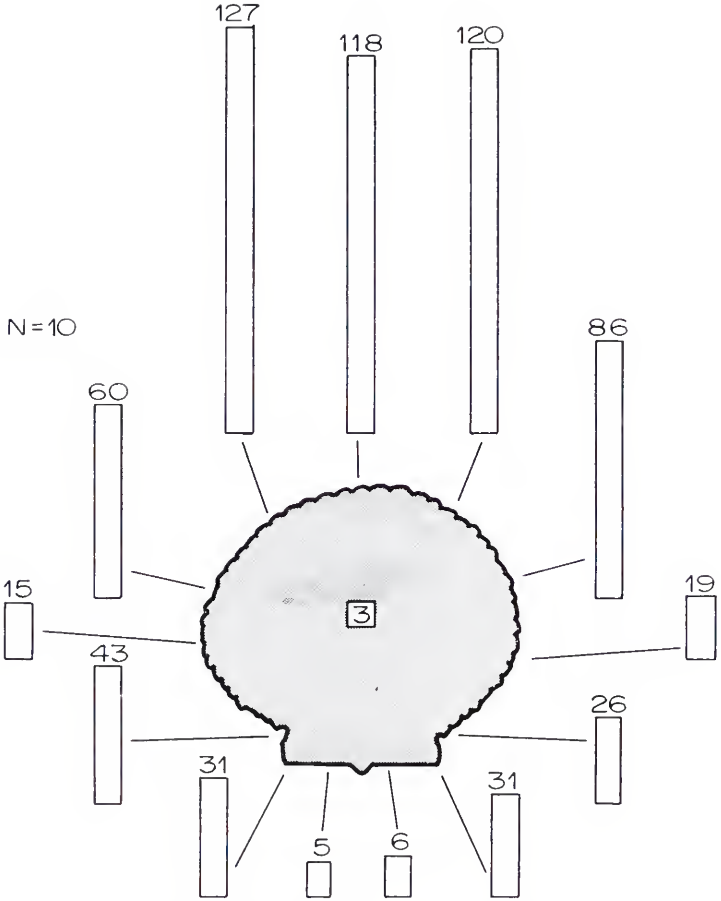


FIGURE 2. Incorporation of ^{14}C-carbonate in regions of the shell. The values show the mean cpm per mg of shell sample for a 4-hour incubation for ten animals. The height of the bars is proportional to incorporation. The medium contained 20 μC/l ^{14}C-bicarbonate.

The calcium-to-carbonate molar uptake ratios for shell edge was determined from the mean values for each time interval indicated in Figures 1A and 1B and are shown in Figure 1B (upper curves). The mean incorporation ratio of all individuals tested was 1.5 ± 0.24 and was not significantly different from 1.00 even at the 50% level for a two-sided Student's t-test. Since a slight increase in molar ratio with time was indicated by the mean values at each point, a regression line of Y on X was calculated from all the individual data points by the method of least squares (Fig. 1B). The correlation coefficient for the plot of molar ratio against incubation time was very low (0.21).

The rate of uptake of ^{45}Ca in mantle of animals maintained in a recirculating sea water tank in Durham, North Carolina was virtually identical with that of animals in running sea water at Beaufort, North Carolina. Incorporation of ^{45}Ca

and ^{14}C-carbonate into shell of animals in recirculating sea water followed a nearly linear course but at a lower rate. An abnormal chalky layer was noted on the inner shell surface near the edge of these animals. Examination of the abnormal layer by scanning electron microscopy indicated that the layer was due to deposition of material different from normal shell and that dissolution of normal shell layers adjoining the chalky deposit was not evident. This deposition may account for isotope incorporation since growth in area at the edge did not occur. Studies described in the following sections were all carried out with animals maintained in running sea water at Beaufort.

Deposition rates in various shell regions

The rate of incorporation of ^{14}C-carbonate varied considerably in different regions of the shell. Figure 2 shows that the highest rates were at the ventral shell edge in the area of the longest ribs and that the lowest rates were in the central shell region and at the dorsal edge adjacent to the hinge. For making measurements of maximum sensitivity, the region of the ventral edge was selected for subsequent studies.

Rate of deposition and shell weight

The rate of deposition of ^{45}Ca and ^{14}C-carbonate at the ventral shell edge as a function of valve weight for one experiment is given in Figure 3. There was a virtual absence of trend in deposition rate with size. The correlation coefficient calculated was −0.22 for ^{14}C-carbonate and −0.13 for ^{45}Ca. Other data on deposition rates of ^{45}Ca and ^{14}C-carbonate over a range of weights from 2 g to 12 g gave the same low correlation. The total number of animals used for these experiments was 131.

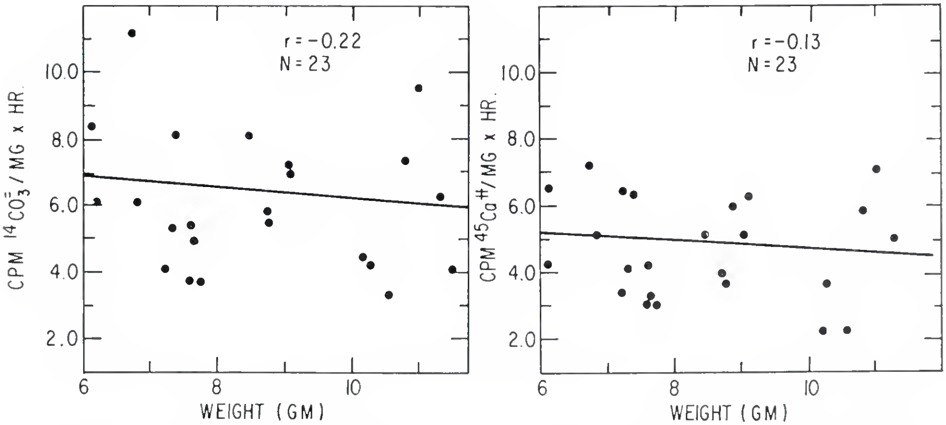


FIGURE 3. Rate of incorporation of ^{14}C-carbonate and ^{45}Ca as a function of weight of the right valve. Each point represents the mean of triplicate pieces from the ventral edge of the shell of one animal. The regression lines were calculated by the method of least squares with r being the correlation coefficient and N, the number of animals.

TABLE I

Diurnal mantle activity and calcification rates.

	10:30 AM–2:30 PM	6:30 PM–8:30 PM
Mantle: Minimum % upturned*	8	83

	10:00 AM–2:00 PM Light	10:00 AM–2:00 PM Dark	7:00 PM–8:30 PM Dark
Calcification cpm ^{14}C-carbonate per mg per hr	24.0 ± 3.0 N = 17	24.3 ± 3.2 N = 25	6.7 ± 1.3 N = 26

* Data from Wrenn (1972). Figures show minimum percentage of animals which turned mantles over the shell edge, forming vertical ridges.

Diurnal deposition rates

Shell growth increments in *A. irradians,* indicated by ridges that delineate a day's growth (Fig. 4), are formed by the upturning of the mantle over the shell edge (Wrenn, 1972; Clark, 1974). Wrenn has demonstrated that the ridges are formed primarily in late afternoon and evening (Table I).

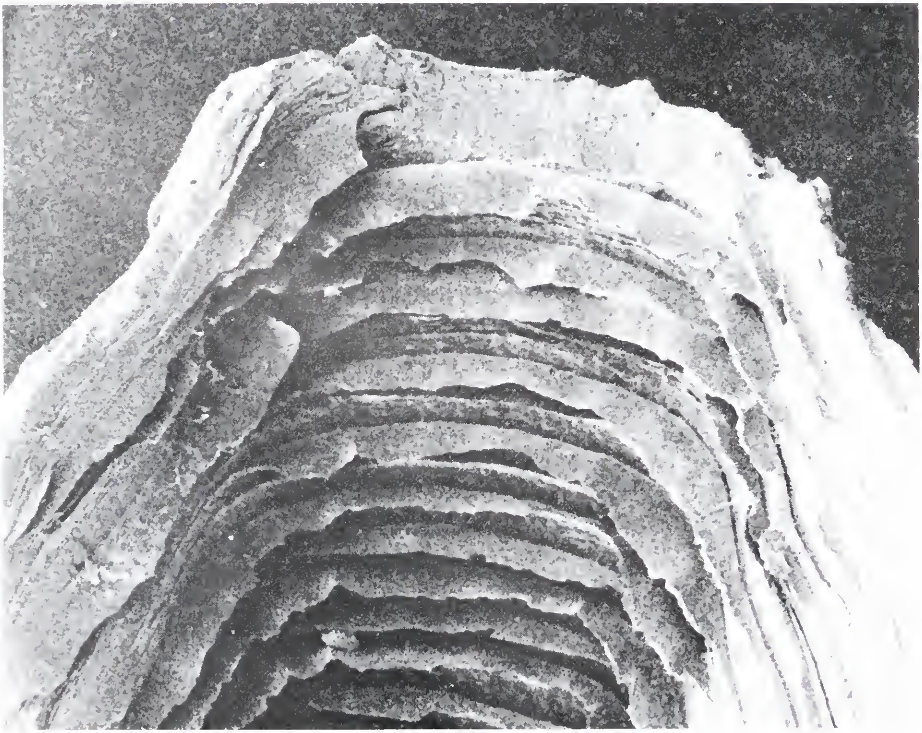


FIGURE 4. Scanning electron micrograph taken at the ventral edge of the right valve (× 30). Each vertically positioned ridge delineates one day's growth.

The rate of incorporation of ^{14}C-carbonate was measured during a period when little ridge formation was occurring and new interridge shell was being deposited (10 AM to 2 PM) and during a period when much ridge formation was taking place (7 PM to 8:30 PM). The rate of ^{14}C-carbonate deposition was more than 3-fold greater when ridges were not being formed as compared with the period of their formation (Table I). The difference was significant at greater than the 99% level for the two-sided Student's t-test.

To test the effect of short-term periods of light and darkness on incorporation rates of ^{14}C-carbonate, scallops were incubated in total darkness and light during the period 10 AM to 2 PM. No significant difference between the two groups was found (Table I).

Protein content of shell

The protein content of the shell ridges was $32.9 \pm 3.9\%$ ($N = 3$), and the protein content of shell including ridges was 16.0 ± 2.1 ($N = 3$). The difference shows that the mineralization of the shell ridges is much less than the other portion of the shell. The average shell protein content of 16% is very high (Wilbur and Simkiss, 1968), probably reflecting in part the elevated protein content of the shell ridges.

DISCUSSION

The measurement of shell growth by means of radioisotopes, especially during short periods, requires that: (1) the shell-forming mantle tissue come into steady state with the medium rapidly (Wilbur and Jodrey, 1952); (2) that shell dissolution by metabolites be minimal as compared with calcium deposition (Crenshaw and Neff, 1969); and (3) that exchange between the shell and the extrapallial fluid in contact with the inner shell surface be at a low level as compared with calcium deposition.

The present study with *Argopecten* has demonstrated that the mantle reaches a steady state with ^{45}Ca of the sea water medium within two hours, a value nearly identical with that of the oyster *Crassostrea virginica* (Jodrey, 1953) and the oyster *Crassostrea gigas* (Kado, 1960). The relatively rapid rate of ^{45}Ca penetration and the resulting small lag in shell incorporation satisfies the steady state requirement for short-term measurements of shell growth with ^{45}Ca. Since the calcium-to-carbonate molar ratio of incorporation is largely independent of time of incubation, the saturation of mantle for ^{14}C-bicarbonate probably takes place at about the same rate as for ^{45}Ca and thus also satisfies the steady state requirement for measurement of short-term mineralization.

Wilbur and Jodrey (1955) suggested that metabolic CO_2 may play an important role in shell carbonate formation in the oyster *Crassostrea virginica*. In *Argopecten*, bicarbonate entering directly from sea water rather than being derived from metabolic CO_2 may be the main source of shell carbonate. This is indicated by a calcium-to-carbonate molar ratio of approximately unity throughout the 5-hour period of exposure to ^{45}Ca and ^{14}C-bicarbonate. If metabolic CO_2 were important, then one would expect the ^{45}Ca- to ^{14}C-carbonate ratio would be higher at the start of the experiment when little metabolizable substrate would be labelled and then decrease with time as ^{14}C-bicarbonate labelled the substrates. However, if there were a rapid turnover in CO_2 fixation in the mantle, bicarbonate from the medium could not be readily distinguished from bicarbonate originating from metabolic CO_2. Hammen and Wilbur (1959) suggested for oyster mantle that pools of organic and amino acids with turnover times in the same range as suggested here for bicarbonate were available for CO_2 fixation from a $H^{14}CO_3$-labelled medium. However, the relative sizes of pools of free bicarbonate and bicarbonate that had been fixed were not determined so an estimate of ther relative contribution to formation of shell carbonate cannot be made. In the absence of data, the relative contribution of bicarbonate from the medium and from metabolic sources must be left open.

The second requirement for the use of radioisotopes in shell growth studies, *i.e.*, that shell dissolution due to organic acid accumulation be minimal, is not likely to be important for *Argopecten* since its valves are normally open and water is circulated over the mantle continuously. This behavior contrasts with that of the clam *Mercenaria mercenaria* studied by Dugal (1939) and Crenshaw and Neff (1969) in which there are periods of valve closure and absence of pumping during which organic acids accumulate in contact with the inner shell surface.

The third requirement, namely the low level of exchange between shell and the extrapallial fluid in contact with the inner shell surface, appears to be met even though exchange is not measurable *in vivo*. Exchange as measured in sea water with $H^{14}CO_3$, and ^{45}Ca in *Argopecten* was 5% to 8% of the total radioisotope incorporation measured at the shell edge at five hours. Exchange *in vivo* may well be lower than this since the amount of ^{14}C-carbonate incorporated in the central region of the valve was less than 3% of that incorporated at the ventral edge (Fig. 2). An exchange rate considerably higher than that in sea water is unlikely in view of the similarity of the concentrations of calcium and carbonate in sea water and the extrapallial fluid of other bivalves (Crenshaw, 1972). A further consideration of the magnitude of exchange relative to net shell growth is the fact that *Argopecten* is forming shell very rapidly as indicated by the size of the daily growth increments.

Even though all the foregoing requirements are fulfilled, caution must still be exercised in interpreting isotope incorporation as a measure of *normal* growth. A case in point relates to specimens of *Argopecten* kept in a recirculating sea water tank rather than in running sea water. These animals deposited both ^{45}Ca and ^{14}C-carbonate yet did not show linear shell growth, nor did they form daily ridges.

The rate of isotope incorporation in molluscan shells has been found to decrease with shell weight (Zischke, Watabe and Wilbur, 1970). However, in *Argopecten* the rate of ^{14}C-carbonate deposition at the edge was not greatly different in shells differing markedly in weight. This may in part be due to the limited range of age of animals available for the studies and in part to the specific nature of growth at the shell edge. The lack of correlation between shell weight and isotope incorporation facilitates the interpretation of isotope data by eliminating excess scatter and unintentional bias due to size of experimental animals.

The observed lower rate of mineral deposition in the evening as compared with midday demonstrates a diurnal control of mineralization by the mantle. The lower rate is correlated with the time of upturning of the mantle and the formation of the daily ridges of the shell (Wrenn, 1972). The protein-to-mineral ratio is increased at the time of ridge formation, as shown by protein analysis. The reduction in mineral deposition may result from (1) a decreased supply of calcium due to a decreased mantle circulation when the mantle muscle contracts, causing upturning; (2) a decreased movement of calcium across the mantle; or (3) a decreased secretion of protein participating in crystal nucleation. The increased protein content of the shell ridges reflects a decrease in mineral deposition by the mantle as shown by isotope incorporation and may involve an increase in protein secretion by the mantle as well. A study of the rate of deposition of labelled protein in the shell at various times of day would resolve this and would indicate whether the mantle exerts a diurnal control of protein secretion as well as a diurnal control of mineralization.

The factors inducing the diurnal variation of mineralization in *Argopecten* are not clear. Short-term lighting does not appear to be a factor since the rates of mineralization in light and darkness at the same time of day were nearly identical. These results are in agreement with those of Dodd (1969), who observed no effect of light on ^{45}Ca deposition in *Argopecten* and other bivalves. However, the timing of ridge formation in *Argopecten* has been shown to be influenced by photoperiod (Wrenn, 1972). Tidal variations can scarcely be a controlling factor since ridge formation occurs at about the same time each day (Wrenn, 1972). Control by daily fluctuations in phytoplankton concentrations associated with tidal changes (Kirby-Smith, 1970) to which the experimental animals would have been exposed is unlikely for the same reason. From the evidence available, we support the suggestion of Dodd (1969) that diurnal growth markings may be brought about by an endogenous rhythm. In *Argopecten,* the rhythm involves a difference in the rate of mineral deposition correlated with an upturning of the mantle during one period of the day.

We thank Dr. Miles A. Crenshaw and Dr. John R. Gregg for their discussion and helpful suggestions in the preparation of the manuscript, and Miss Mayme Lee Blankenship for taking the electron micrograph. A portion of the experimental work was carried out at the Atlantic Estuarine Fisheries Center (National Marine Fisheries Service), Beaufort, North Carolina. We appreciate the help of Dr. T. R. Rice for making space and facilities available. This study was supported in part by grant DE 01382-12 from the National Institute of Dental Research.

SUMMARY

1. Incorporation of calcium and carbonate into shell has been studied in the scallop *Argopecten irradians* using ^{45}Ca and ^{14}C-bicarbonate.

2. The incorporation of ^{45}Ca and ^{14}C-carbonate into shell was linear with time after a lag period of 1 to 2 hours. The shell-forming mantle tissue attained a steady state with respect to ^{45}Ca in the sea water medium within 2 hours.

3. The molar ratio of ^{45}Ca to ^{14}C-carbonate deposited in shell was not significantly different from unity during 5 hours.

4. The rate of incorporation of ^{14}C-carbonate into shell was highest at the ventral edge and extremely low in the central and hinge areas.

5. The rate of incorporation at the ventral shell edge did not change with increase in shell size.

6. The rate of incorporation of carbonate was low at night when growth ridges form and increased 3-fold at midday when growth ridges are not being formed.

7. The protein content of the shell ridges was 32.9 ± 3.9% and the protein content of the shell including ridges was 16.0 ± 2.1%.

LITERATURE CITED

BRAY, G. A., 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.*, **1**: 279–285.

BROWN, F. A., JR., M. F. BENNETT, H. M. WEBB, AND C. L. RALPH, 1956. Persistent daily, monthly, and 27 day cycles of activity in the oyster and quahog. *J. Exp. Zool.*, **131**: 235–262.

CLARK, G. R., II, 1968. Mollusk shell: daily growth lines. *Science*, **161**: 800–802.

CLARK, G. R., II, 1974. Calcification on an unstable substrate: marginal growth in the mollusk *Pecten diegensis*. *Science*, **183**: 968–970.

CRENSHAW, M. A., 1972. The inorganic composition of molluscan extrapallial fluid. *Biol. Bull.*, **143**: 506–512.

CRENSHAW, M. A., AND J. M. NEFF, 1969. Decalcification at the mantle-shell interface in molluscs. *Amer. Zool.*, **9**: 881–885.

DODD, J. R., 1969. Effect of light on rate of growth of bivalves. *Nature*, **224**: 617–618.

DUGAL, L. P., 1939. The use of calcareous shell to buffer the product of anaerobic glycolysis in *Venus mercenaria*. *J. Cell. Comp. Physiol.*, **13**: 235–251.

HAMMEN, C. S., AND K. M. WILBUR, 1959. Carbon dioxide fixation in marine invertebrates. I. The main pathway in the oyster. *J. Biol. Chem.*, **234**: 1268–1271.

HOUSE, M. R., AND G. E. FARROW, 1968. Daily growth banding in the shell of the cockle, *Cardium edule*. *Nature*, **219**: 1384–1386.

JODREY, L. H., 1953. Studies on shell formation. III. Measurement of calcium deposition in shell and calcium turnover in mantle tissue using the mantle-shell preparation and Ca^{45}. *Biol. Bull.*, **104**: 398–407.

KADO, Y., 1960. Studies on shell formation in mollusca. *J. Sci. Hiroshima Univ., Ser B*, **19**: 163–210.

KIRBY-SMITH, W. W., 1970. Growth of the scallops, *Argopecten irradians concentricus* (Say) and *Argopecten gibbus* (Linné), as influenced by food and temperature. *Ph.D. dissertation, Duke University*, 127 pp.

RHOADS, D. C., AND G. PANNELLA, 1970. The use of molluscan shell growth patterns in ecology and paleoecology. *Lethaia*, **3**: 143–161.

SANDEEN, S. I., G. C. STEPHENS, AND F. A. BROWN, JR., 1954. Persistent daily and tidal rhythms of oxygen consumption in two species of marine snails. *Physiol. Zool.*, **27**: 350–356.

SPEEG, K. V., JR., AND J. W. CAMPBELL, 1968. Formation and volatilization of ammonia gas by terrestrial snails. *Am. J. Physiol.*, **214**: 1392–1402.

WILBUR, K. M., 1972. Shell formation in mollusks. Pages 243–282 in M. Florkin and B. T. Scheer, Eds., *Chemical Zoology, Vol. VII*. Academic Press, New York.

WILBUR, K. M., AND L. H. JODREY, 1952. Studies on shell formation. I. Measurement of the rate of shell formation using Ca^{45}. *Biol. Bull.*, **103**: 269–276.

WILBUR, K. M., AND L. H. JODREY, 1955. Studies on shell formation. IV. The respiratory metabolism of the oyster mantle. *Biol. Bull.*, **108**: 346–358.

WILBUR, K. M., AND K. SIMKISS, 1968. Calcified shells. Pages 229–295 in M. Florkin and E. H. Stotz, Eds., *Comprehensive Biochemistry, Vol. 26A*. Elsevier Publishing Co., Amsterdam.

WRENN, S. L., 1972. Daily increment formation and synchronization in the bay scallop. American Society of Zoologists Summer Meeting, Division of Comparative Physiology and Biochemistry with the American Physiological Society, *Amer. Zool.*, **12**: xxxvii.

WRIGHT, D. G. S., 1971. Aspects of respiration of the fresh water snail *Lymnae pallustris* (Müller). *Can. J. Zool.*, **49**: 997–1000.

ZISCHKE, J. A., N. WATABE, AND K. M. WILBUR, 1970. Studies on shell formation: measurement of growth in the gastropod *Ampullarius glaucus*. *Malacologia*, **10**: 423–439.

Reference: *Biol. Bull.*, **148**: 483–496. (June, 1975)

AGONISM IN ASTEROIDS

DON R. WOBBER

Biology Department, San Francisco State University, San Francisco, California

In general sea stars have been said to be "slow-moving creatures that lie or cling to the substrate most of the time, and move about only intermittently to search for food or to avoid some physical stress" (Feder and Christensen, 1966, page 92). In regard to the sea stars *Pisaster ochraceus* and *Leptasterias hexactis*, Menge and Menge (1974, page 208) state, "It is not surprising that intraspecific aggression is nonexistent with these behaviorally simple animals." However, sea stars do pursue and catch moving prey, dig for clams, feed on particulate matter, lurch forward onto prey, or actively defend themselves against predation or cannibalism (Mauzey, Birkeland, and Dayton, 1968). Moreover, sea stars, like other echinoderms, may be capable of complex "social behavior." Reese (1966), defining social behavior as interactions between individuals as opposed to "non-social behavior" resulting from individual interaction with the physical environment, concluded that echinoderms are not capable of social behavior other than that related to reproductive activities. This conclusion was challenged by Brun (1969), and Warner (1971) with respect to brittlestar aggregations; by Dix (1969), and Pearse and Arch (1969) regarding urchin aggregations; and by Branham, Reed, Bailey and Caperon (1971), and Goreau (1964) regarding sea star distribution. Goreau (1964, page 25) thought *Acanthaster planci* to be territorial in distribution, noting "a brief transitory association" of two individuals on two occasions. Although the above authors indicate the existence of echinoderm social behavior not related to reproductive activities, only Pearse and Arch (1969) describe specific interactions between individuals that might contribute to such social behavior.

Intraspecific agonistic encounters involving ray contact between individual sea stars may reflect complex social behavior. Such encounters, here referred to as bouts, have been observed in Monterey Bay, California in all three living orders of asteroids: Forcipulata (*Pycnopodia helianthoides, Leptasterias hexactis*, and *Pisaster giganteus*); Spinulosa (*Patiria miniata*); and Phanerozonia (*Dermasterias imbricata* and *Mediaster aequalis*), and interspecific bouts were seen between *Pycnopodia* and two species of *Pisaster, P. giganteus* and *P. brevispinus* (Wobber, 1974). Sea star bout behavior therefore may be widespread, perhaps affecting the way many species feed and are distributed. This paper describes the agonistic bouts as seen in the sea stars *Patiria* and *Pycnopodia*.

MATERIALS AND METHODS

Field observations resulting from about 240 hours of scuba diving between 1971 to 1974 were done in a 75 × 40 m study area on the seaward side of the U. S. Coast Guard breakwater at the southwest end of Cannery Row, Monterey, California. Depths ranged from 3–19 m. Observations were made on the large boulders forming the breakwater, and on the gently sloping sand bottom at its base as well as in aquariums. Undersea observations were recorded on plastic writing boards and by still or motion pictures, the latter augmented with time lapse series analyzed frame-by-frame (see Wobber, 1974, for further details).

Bouts were initiated by placing fresh but dead market squid, *Loligo opalescens*, a natural food for sea stars in the study area (Wobber, 1973), on the reef in weighted net bags and allowing sea stars to converge upon it; by squirting beef broth (Swanson brand) between two stationary sea stars; or by placing sea stars within 1 cm of one another.

Sex determination

Gonads from pairs in a bout were inspected to determine sex, testes were identified by their pale color, and ovaries by their pink or orange color. Since *Patiria* show an ill-defined breeding period and seem to spawn at all times of the year (Farmanfarmaian, Giese, Boolootian and Bennett, 1958), surveys of this species during May and June were considered typical distributions, not influenced by any type of sexual aggregation.

Sensitivity to ray contact

Stationary sea stars in a natural state were touched on various parts of the rays and aboral surface by diver-held sea stars and reactions of the "contacted" animals noted.

Bout outcome determination

Specimens of *Patiria* placed within 1 cm of one another will move toward one another and engage in bouts. A series of experiments was designed in which two sea stars were moved to a different, but nearby, location, placed within 1 cm of one another, and bout outcomes recorded; starting positions were marked with plastic pegs.

Convergent feeding

To determine how sea stars approach and feed upon large food items, a series of tests, termed target tests, were made on the sandy bottom at 19 m on a plastic tarpaulin which had been marked by painting 4 concentric circles at 20 cm intervals. Bait was placed within the center circle and varying numbers of conspecific individuals spaced at equidistant intervals around the outer edge of the largest circle. Each test ran 22 minutes.

RESULTS

Intraspecific bouts, Patiria miniata

Bouts between specimens of *Patiria* seemed related to food gathering, but took place even when feeding was not obvious. The mean duration time of 52 timed bouts was 28 minutes with a standard deviation of 24.5 minutes, and a range of 3–120 minutes. On rare occasions bouts lasted more than two hours.

Ray movements during bouts can be described by the following motions or positions: (1) *extracting,* the withdrawal of a ray from beneath the opponent without a general withdrawal of the whole animal; (2) *lifting,* the raising of a ray to a position above the aboral surface of the opponent; (3) *holding,* the holding of a ray in a position above the aboral surface of the opponent; (4) *feinting,* the slight lowering and raising of the ray when in the holding position; (5) *arching,* the holding of a ray, further back than in the holding position, in a position above the sea star's own aboral surface, often with a ray in an 'S' confirmation (Fig. 1); (6) *dropping,* the lowering of a ray toward the opposing sea star; (7) *reaching,* the stretching of a ray out onto the aboral surface of the opposing sea star after a dropping motion; (8) *pushing,* the forcing of the distal end of a ray against the distal end of the ray of the opponent, often folding the tip of the ray over the opponent's ray (Fig. 2); and (9) *locking,* the surrounding and pressing in of two rays of one animal on one of the other animal's rays (Fig. 3). Two or more specimens of *Patiria* in locking positions often share the same food item.

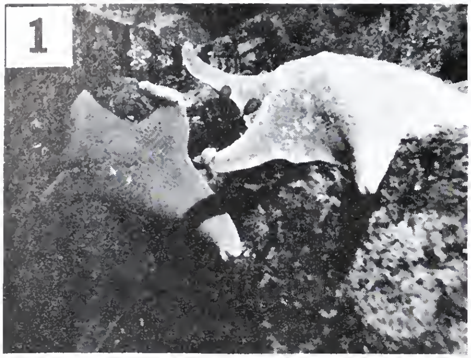
The above activities are not necessarily independent acts, but describe parts of what can be a continuous motion, graceful, fluid, and variable, and possibly dependent upon the reactions and position of the opposing sea star. It was common to see specimens of *Patiria* with facing rays in holding positions, poised without contacting one another, for long intervals. Two specimens of *Patiria* with facing rays in holding positions remained completely out of physical contact for 7.7 minutes, although ray contact both preceded and followed this period of non-contact.

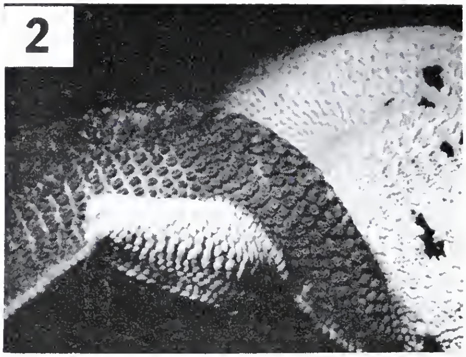
Holding or arching and the time and height of these positions often determined ray dominance: once contact was resumed the last ray to drop generally landed on top of the ray or body of the opponent, an apparent advantage. When animals were out of contact, the holding position was often lower than when in contact, sometimes even horizontal.

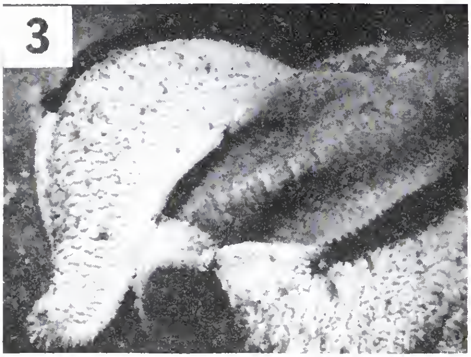
Bouts were seen to terminate by withdrawal of one or both animals, or locking or settling down near one another, often with rays overlapped, the latter taking place only when food items were involved. Bouts continued throughout feeding. Extensive pursuit in this species was not noted.

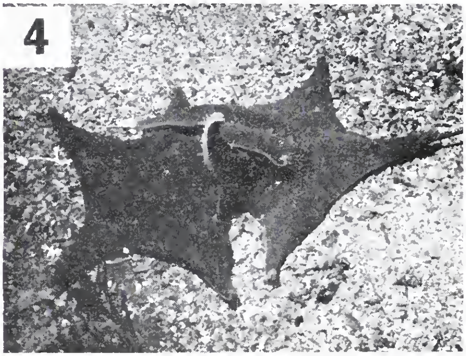
Two distinct types of bouts between specimens of *Patiria* were recorded: (1) *continuous-contact,* during which the animals maintain contact throughout, engaging multiple rays and moving their bodies against one another (Fig. 4) and, if one animal is pushed off balance or otherwise disadvantaged, the other generally follows up by actions such as a dominant two-ray advance (Fig. 5), which initiates opponent withdrawal; and (2) *intermittent-contact,* during which the animals maintain bout positions although out of contact much of the time (Fig. 1). Intermittent-contact bouts showed a tendency toward non-contact in the later stages. In five such bouts, pairs were out of contact with one another a combined mean of 51.5% of the time during the first half of the bouts and 78.5% of the time during the second half (Table I).

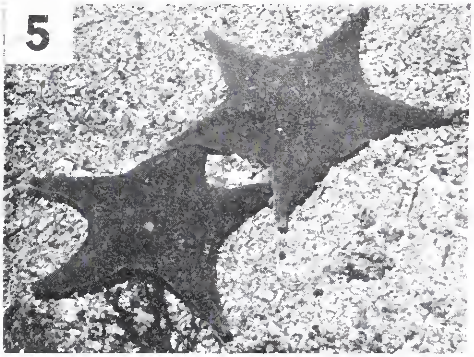
During intermittent-contact bouts facing rays often touched one another briefly, the aboral surface of one sea star's ray tip contacted by the oral surface of the other sea star's ray tip. This contact initiated a swift raising of the rays of both individuals, the ray touched on its aboral surface going into the arching or holding position, the ray of the opponent assuming a lower hold position (Fig. 1). Within











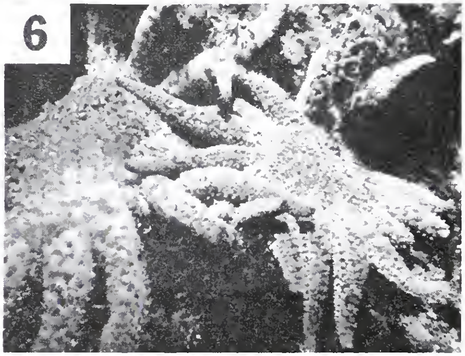


FIGURE 1. Specimens of *Patiria* engaged in an intermittent-contact bout; sea star on left with facing ray in arching position, sea star on right with facing ray in holding position.

FIGURE 2. Specimens of *Patiria* showing the detail of rays in the pushing position during an intermittent-contact bout.

FIGURE 3. Specimens of *Patiria* in locking position; sea star on right with two rays surrounding one ray of sea star on the left.

FIGURE 4. Specimens of *Patiria* engaged in a continuous-contact bout.

FIGURE 5. Specimens of *Patiria* engaged in continuous-contact bout; sea star at right in two ray dominant advance, sea star on left withdrawing.

FIGURE 6. Specimens of *Pycnopodia* engaged in intraspecific bout.

TABLE I

Non-contact time within five Patiria miniata *intermittent-contact bouts recorded by time lapse motion pictures.*

Bout	Duration in minutes	% non-contact time		
		Total	First half	Second half
1	90	53.5	28.5	78.7
2	84	80.8	67.4	94.2
3	72	88.5	79.5	97.8
4	20	20.9	9.6	32.2
5	17.4	81.0	72.4	89.7
$\bar{x} \pm$ s.d.	56.7 ± 35.3	64.9 ± 28.0	51.5 ± 30.7	78.5 ± 26.9

the 5 bouts shown in Table I, a combined total bout time of 283.4 minutes, such a sequence was seen 35 times ($\bar{x} = 7.0 \pm 2.9$ s.d., range 2–9). Termination of intermittent-contact bouts usually followed the pattern of a dominant two-ray advance which appeared to initiate opponent withdrawal (Fig. 5).

Primary ray positions of bouts were classified into three categories: advantageous; neutral; and disadvantageous, depending on the ray's relationship to the opponent's facing ray(s), *i.e.,* in a dominant position the ray was either touching the opponent's aboral surface or in a higher position than the opponent's ray(s), and thus able to descend on top of the opponent. Holding positions were separated into three categories depending upon the ray's position relative to the horizontal: high hold, 60° to 90°; medium hold, 30° to 60°; and low hold, from horizontal or below to 30°. Acts such as reaching and feinting were not considered in these data. Intra-individual sequence patterns of the above acts were taken from time lapse motion pictures of three intermittent-contact bouts of *Patiria* and translated into frequencies of occurrence (Table II, Fig. 7). One action sequence was the subordinate ray touch which led to an arching position 69% of the time or a high hold position 31% of the time. Trends in a given bout toward overall dominant-subordinate relationships of individual sea stars were not established. Cannibalism was not observed in *Patiria.*

Specimens of *Patiria* in intermittent-contact bouts can engage two opponents simultaneously and cause withdrawal of both.

Intraspecific bouts, Pycnopodia helianthoides

Bouts occurred when two sea stars came in contact, whether feeding or not. Although no quantitative data were taken of approximately 50 bout observations, durations of most were estimated to be less than two minutes. The longest observed bout lasted about 10 minutes.

A ray movement position unique to *Pycnopodia* is *side-slipping,* the semi-horizontal sliding of rays between, then over and onto the rays of the opponent, sometimes accomplished by a rotating of the whole body clockwise or counterclockwise. *Pycnopodia* did not engage in feinting, pushing, or locking positions. Up to seven rays of one sea star may be involved in one bout at a given time.

TABLE II

Frequencies of intra-individual two-act agonistic sequences from intermittant-contact bouts of three Patiria *pairs where one ray each of each pair of sea stars was involved. Observed frequencies in each row are figures on top, figures below in parentheses indicate percent occurrence of following act. (Columns add to 100 ±).*

Following Act	INITIAL ACT										
	Advantageous				Neutral			Disadvantageous			
	Arching	Dominant ray touch	High hold*	Base ray contact**	Pushing	Both base ray contact	Extracting	Subordinate ray touch	Medium hold††	Low hold†	Base ray contacted
Arching	0	0	1 (02)	3 (16)	0	1 (20)	0	20 (69)	1 (04)	1 (06)	3 (38)
Dominant ray touch	9 (29)	0	15 (34)	0	0	0	0	0	2 (08)	3 (17)	0
High hold	1 (03)	16 (55)	0	6 (32)	3 (50)	0	1 (33)	9 (31)	7 (29)	1 (06)	2 (25)
Base ray contact	7 (23)	1 (03)	3 (07)	0	2 (33)	1 (20)	1 (33)	0	3 (13)	1 (06)	0
Pushing	2 (06)	0	1 (02)	1 (05)	0	0	0	0	0	2 (11)	0
Both base ray contact	1 (03)	0	0	1 (05)	0	0	1 (33)	0	1 (04)	0	0
Extracting	0	0	0	0	0	1 (20)	0	0	0	0	3 (38)
Subordinate ray touch	2 (06)	0	12 (27)	1 (05)	0	0	0	0	6 (25)	7 (39)	0
Medium hold	4 (13)	9 (31)	5 (11)	5 (26)	0	0	0	0	0	1 (06)	0
Low hold	5 (16)	3 (10)	5 (11)	1 (05)	0	2 (40)	0	0	2 (08)	0	0
Base ray contacted	0	0	2 (05)	1 (05)	1 (17)	0	0	0	2 (08)	2 (11)	0

*Disadvantage 57.7% **Disadvantage 37% †Advantage 22% ††Advantage 21%

Initial ray touching, extracting, lifting, holding, arching, dropping, and reaching are part of the bout, but ray movements are faster than the ray movements of *Patiria*.

In most bouts observed, initial contact was a touching of the distal ends of the rays by both individuals, followed by a pulling upwards or backwards of the contacted rays. An approaching sea star sometimes hesitated and raised its rays, or slid its rays over or between the facing rays of the other animal. Individuals have not been seen to move completely on top of one another. Once initial contact was made, the body of a sea star generally maintained its position as it withdrew one or more rays at a time and placed them on the aboral surface of the other sea star. It was not unusual to see both individuals engaged in extracting rays in this manner and then laying them down on one another's surface simultaneously.

During bouts, sea stars were in continuous contact with one another (Fig. 6). Neither pedicellariae nor tube feet were used for adhering to or defending against the opponent. Withdrawal appeared to be initiated by the extent to which one or more rays were successfully placed on the aboral surface of the opponent: the closer to the center of the disc, the greater the reaction. Bout outcomes resulted in the withdrawal of one or both animals, or the pursuit of one by the other. Pursuit distances up to 3 m were recorded. If only one individual withdrew, the withdrawn animal sometimes stopped 10–100 cm away, raised the rays which were facing the other animal and approached directly again. The returning sea star immediately initiated another bout by moving its raised facing rays onto the opponent's aboral surface. Bouts between specimens of *Pycnopodia* did not terminate in overlapping or settling down in contact with one another, even over large items of food.

An individual may engage two or more opponents in bout behavior, employing several groups of rays around its periphery simultaneously.

Interspecific bouts

Interspecific bouts were seen between specimens of *Pycnopodia* and specimens of two species of *Pisaster*, *P. giganteus* and *P. brevispinus*. Five such bouts involved a specimen of *Pycnopodia* attacking a feeding specimen of *Pisaster* sp. Six additional bouts were initiated by feeding dead *Loligo opalescens* to either species of *Pisaster*. In all bouts a specimen of *Pycnopodia* approached and placed some of its facing rays on the aboral surface of the feeding *Pisaster* individual, removing the rays almost immediately in a continuous "flailing" motion, while reaching beneath the feeding sea star with other rays, often thus obtaining some or all of the food. The attacking animal's rays were fastened onto by the pedicellariae of the specimen of *Pisaster* which raised and twisted its facing rays causing the aboral surface bearing the pedicellariae to come down on the aboral surface of the attacking animal. The specimen of *Pycnopodia* repeatedly withdrew its rays, but did not use its own pedicellariae. These encounters lasted approximately 30 seconds. When the *Pycnopodia* individual withdrew, its facing rays were sometimes shortened and thickened, the tips of many curled back over themselves, appearing temporarily immobile, as though paralyzed. On two separate occasions a specimen of *Pisaster giganteus* feeding on a large dead fish was approached by a specimen of *Pycnopodia* and the animals engaged in a bout. Both times the specimen of *Pycnopodia* withdrew although one specimen of *Pycnopodia* was 40 cm in diameter and its opponent 28 cm in diameter.

Specimens of *Patiria* were often touching or being touched by specimens of *Pycnopodia* or*Pisaster* sp., either incidentally or on food, but no interspecific agonism was observed.

Sex determination

Sex differences between 19 arbitrarily chosen pairs of *Patiria* in a bout did not show significant deviation from chance ($P > 0.70$, d.f. = 2), therefore bouts are apparently not a sexual behavior. As *Pycnopodia* populations were sparse, gonadal material was not checked in this species.

Sensitivity to ray contact

Hand-held specimens of 10 *Pycnopodia* 6 cm in diameter touched to the aboral surface of 10 stationary animals 45 cm in diameter initiated no withdrawal responses, whereas 10 specimens of the same or larger size initiated withdrawal in all cases, the response appearing more intense as the stationary animal was touched closer to the center of the disc. Touching on the madreporite did not elicit unusual withdrawal responses. Reactions of specimen pairs of *Patiria* tested in the above manner were so slow that results were uncertain.

Bout outcome determination

The following observations were performed only with specimens of *Patiria* as any handling or manipulation of specimens of *Pycnopodia* appeared to affect subsequent responses.

Size. Nine pair of sea stars were selected; one member of each pair being over 16 cm and one being under 13 cm in diameter. Each pair was placed with rays closest to the madreporite opposing one another. Of the nine bout outcomes, four large and five small specimens of *Patiria* withdrew; tested size differences therefore were not significant ($P > 0.70$, d.f. = 1).

Madreporite position. Opponent madreporites were not directly attacked, nor did the touching of a madreporite by an opponent initiate withdrawal. However, of 21 pairs tested for bout outcomes, 18 with the madreporite toward the opponent and 3 with the madreporite away from the opponent withdrew. The Chi-square value of 10.72 ($P < 0.005$, d.f. = 1) indicates that madreporite position is a highly significant factor.

Area recognition. Two experiments were designed to determine whether *Patiria* individuals might have a behavioral bout advantage by virtue of "recognition" of the area beneath them (thus indicating territoriality).

In the first experiment, two animals of similar size were moved at the same time, the same approximate distance through the water. One, the "replaced" sea star, was set down on the same spot from which it had been removed; the other, the "transported" sea star, was placed 1 cm from the closest ray of the replaced animal, positioned madreporite-to-madreporite. Of 14 trials, only one replaced sea star withdrew. Chi-square was highly significant ($P < 0.005$, d.f. = 1) and territoriality is strongly supported.

In the second experiment, to determine whether the obvious advantage to the replaced sea star was due to area recognition or related to disturbed sand on which the animal was placed, two specimens of *Patiria* of similar size were moved as above, but in this series, one animal was placed on the area from which the other sea star had just been lifted, and the other placed alongside. Out of 9 trials, 5 animals placed alongside the disturbed area and 4 placed on the disturbed area withdrew (Chi-square = 0.12, $P > 0.70$, d.f. = 1), suggesting that the disturbed area itself is not a factor in the outcome.

Area recognition vs. *size or madreporite position.* In tests of 9 bout pairs where the replaced sea star was either larger or smaller (within a 3 cm variation), all transported animals withdrew. In similar tests of 10 bout pairs, madreporite position being the variable, all transported animals withdrew, indicating a high degree of territoriality regardless of relative body orientation or size variation of the magnitude tested.

Convergent feeding

Three target tests (see materials and methods) were carried out to determin how individuals of *Patiria* converge on large food items. Fresh animals were used for each test. Rays closest to the bait at the start of the tests became the leading rays in movement toward the bait. Specimens of *Patiria* first to arrive at the bait engaged in bouts with one another and approaching individuals, even-

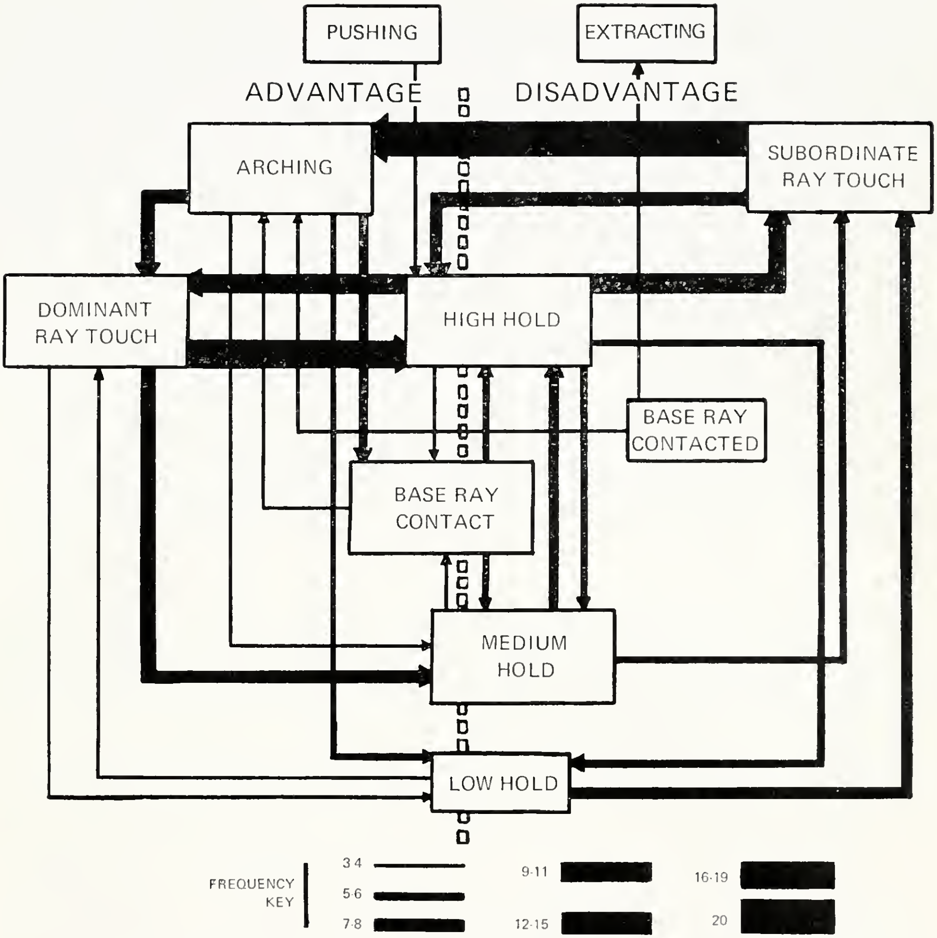

PUSHING
EXTRACTING
ADVANTAGE
DISADVANTAGE
ARCHING
SUBORDINATE RAY TOUCH
DOMINANT RAY TOUCH
HIGH HOLD
BASE RAY CONTACTED
BASE RAY CONTACT
MEDIUM HOLD
LOW HOLD
FREQUENCY KEY
3-4
5-6
7-8
9-11
12-15
16-19
20

Figure 7. An agonistic sequence diagram of *Patiria* intermittent-contact bouts derived from observed frequencies in Table II. Thickness of lines indicates frequency of occurrence, and arrowheads indicate direction of the sequence. Items to the left of the dashed line have a bouting advantage, to the right a disadvantage. Frequency values 1 and 2 were eliminated for simplification.

tually covering the bait and driving latecomers away. After 22 minutes, the number of animals on the bait was approximately the same regardless of the starting group size (Table III). In the starting groups 10, 18, and 26; 90%, 83% and 80.8% respectively either reached the bait or engaged in bouts with animals on the bait. The more animals in the starting group, the more subsequent movement away from the bait by withdrawing animals. Of those withdrawn, 71.7% were recorded as engaged in bouts on the tarpaulin where there was no possibility of food.

TABLE III

Target test results showing dispersal of three variable quantity test groups of Patiria *around a fixed bait after 22 minutes. Figures in parentheses represent sea stars which were not on bait but were engaged in bouts.*

Test	Number of sea stars	Distribution (distance from bait)						Total not on bait	% bouts not on bait
		On bait	0 to 20 cm (not on bait)	20 to 40 cm	40 to 60 cm	60 to 80 cm	Over 80 cm		
1	10	6	1 (1)	2 (2)	—	—	1 (?)	4 (3)	75
2	18	5	4 (4)	4 (4)	2 (2)	—	3 (?)	13 (10)	77
3	26	7	3 (3)	4 (4)	5 (4)	2 (1)	5 (?)	19 (12)	63
		Total % engaged in bouts and not on bait							71.7

Population data

Percentage of sea stars engaged in bouts. Because *Pycnopodia* bouts were rarely seen (less than 1 bout for every 10 hours of diving), no estimate was made of the percentage of bout pairs. *Patiria* bouts were extremely common. Counts

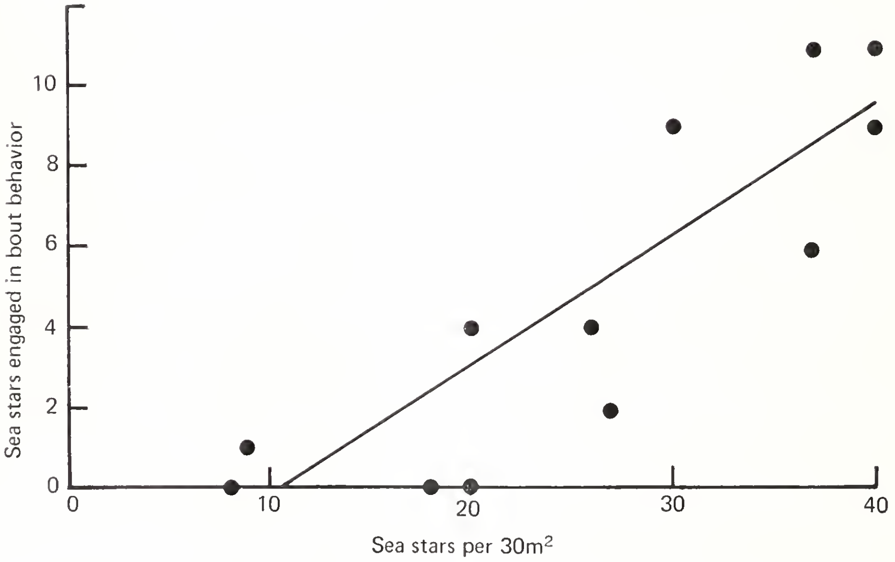


FIGURE 8. Relation of sea star density to bout behavior in 12 2 × 15 transect surveys of *Patiria* ($r = +.86$).

on the exposed surfaces of the large rocks that form the breakwater from three different locations of 30 m^2 at 09:00, 11:00, and 14:00 hours showed that of a total of 389 individuals, a mean of 23.7% were engaged in bouts, s.d. ± 4.09%, range 19.0–26.6%. From this sampling, of the total of those individuals engaged in bouts, 93.7% were engaged in the intermittent-contact type of bout and the remainder were engaged in the continuous-contact type of bout. Although no quantitative data were taken, *Patiria* bouts appear to continue nocturnally at approximately the same level. Twelve 2×15 m transect surveys taken on the sandy bottom in the study area indicated an increase in bout activity with increasing density (Fig. 8).

Dispersion surveys. Subtidal population distribution data was impractical for *Pycnopodia* because of the sparse population (0.013–0.1015 per m^2). *Patiria* dispersion data were taken on the sand at the base of the breakwater during May and June of 1972, from areas where convergent feeding did not seem to play a part. The combined total area of all surveys was 410 m^2. Analysis was made in regard to randomness and the data were fitted to a Poisson distribution, resulting in a Chi-square value of 96.3. The hypothesis that the animals are distributed randomly was rejected ($P < 0.0001$, d.f. $= 2$). Since the ratio $s^2/\bar{x} = 2.43$ is greater than unity, it was concluded that individuals of *Patiria* exhibit contagious distribution.

Discussion and Conclusions

The results demonstrated that agonistic bouts are an important social behavior in regard to *Patiria* and *Pycnopodia.* There are major differences in intraspecific bout behavior between the two species. *Patiria* bouts seemed less aggressive, mostly of the intermittent-contact type where both individuals were out of physical contact for long periods of time, occasionally terminating in food sharing. A withdrawing individual was not pursued extensively. *Pycnopodia* bout outcomes were quick and decisive, the winner maintaining food or area, and sometimes pursuing the loser for distances up to 3 m.

In interspecific bouts between specimens of *Pycnopodia* and *Pisaster* sp., dissimilar behaviors were used by each species. Pedicellariae, used only by specimens of *Pisaster,* seemed to initiate withdrawal of the opponent, temporarily immobilizing its rays, a behavior which allowed specimens of *Pisaster* to compete with the larger, more active sea star, perhaps a more important function for pedicellariae than the occasional capture of food as described by Feder and Christensen (1966) and Robilliard (1971). Menge and Menge (1974) report use of pedicellariae by *Pisaster ochraceus* in aggressive behavior against *Leptasterias hexactis.*

I saw no indications of cannibalism as reported by Mauzey *et al.* (1968) and Greer (1961) for *Pycnopodia.* The non-predatory intraspecific bouts I observed may have evolutionary roots in intraspecific competition rather than in cannibalism. However, Mauzey *et al.* (1968) describes the use of the rays in cannibalistic fighting, so intraspecific bouts cannot be assumed to be a universally harmless means by which sea stars confront one another.

It is not clear why a specimen of *Patiria* with its madreporite closer to the opponent is at a bout disadvantage: the difference in adult size was not significant (although the animal with the longest reach should logically be more of a threat to the opponent's madreporite); the madreporite was not directly attacked nor did the touching of it in itself cause withdrawal. Bout differences regarding body orientation may have more to do with the rays themselves than the relative position of the madreporite: rays adjacent to the madreporite may be more sensitive than other rays and therefore at a disadvantage. Selective advantages to such ray differentials are not apparent. Kjerschow-Agersborg (1922) claims that in *Pycnopodia* the rays adjacent to the madreporite lead during locomotion, a factor that could be related to differential ray sensitivity, however, observations during this study indicated that neither *Pycnopodia* nor *Patiria* rotate the body to change direction nor favor certain rays as leading rays.

Noble (1939) presented a general definition of territory as "any defended area." The animal defending territory usually "wins" by driving away conspecifics (Tinbergen, 1952). My results suggest that a territorial behavioral advantage exists in *Patiria* populations which outranks an advantage in body orientation in regard to ray or madreporite positions.

Woolf (1968, page 283) states that animals exhibiting territoriality follow a uniform rather than a contagious distribution pattern. The contagious distribution demonstrated by populations in the study area may be due to the length of time spent in individual bouts and the high per cent of sea stars so engaged, even though bout outcomes may finally result in spacing-out. Target tests illustrated this (Table II). Specimens of *Patiria* converged on the bait; however, once it was covered, late comers withdrew. The majority of the withdrawn animals did not disperse evenly but, at least temporarily, engaged in bouts with one another, even in the absence of food.

Pycnopodia bouts generally terminated rapidly with one or both animals withdrawing, even over food; therefore, bout behavior appeared to space-out individuals of this species.

Bout abilities may be selected for in competitive situations because the winner retains possession of the food, grows larger, uses less energy in food search, better survives when food is scarce, and thus produces more progeny. The spreading effect of the sea stars which withdraw from bouts may result in all the individuals of the population utilizing food more evenly.

Continuous-contact *Patiria* bouts appeared to require a large degree of individual effort. As up to 26.6% of the individuals in a *Patiria* population were engaged in bouts, it would not be surprising that intense forms of this activity would tend toward modification. Modification to intermittent-contact would have certain advantages: (1) lower energy utilization; (2) the possibility of feeding while engaged in a bout; (3) the ability to bout with two animals simultaneously (perhaps impossible in continuous-contact bouts in this species); and (4) quick withdrawal from the bout by either animal at any time.

Within intermittent-contact bouts, contact decreased with time, pointing to mechanisms which reduce the effort involved over time, such as habituation or short term learning as demonstrated in *Pisaster giganteus* by Landenberger (1966). A complex level of communication may be indicated both by a progression toward less contact, and by common sequence patterns which elicit specific changes in the behavior of recipient animals (Dingle, 1969). Differences within the two types of bouts, such as change of intensity, exaggeration of components (*e.g.,* in intermittent-contact bouts, pushing with the distal ends of rays in contrast to whole body pushing in continuous-contact bouts), and other factors, imply a pattern of signal movements derived through a process of ritualization, as reviewed by Blest (1961). Blest (1961, page 102) hypothesized that, "in the course of evolution, both locomotory movements and acts (concerned with comfort, heat-regulation, and capture of prey) have been selected and modified to produce signals. Such movements have been termed 'derived' and may exist alongside their ancestral activities." The evolutionary development by which such movements have arisen has been called "ritualization" by Tinbergen (1952).

I am deeply grateful to Robert D. Beeman, William J. Light, Jack T. Tomlinson, and Albert Towle, San Francisco State University; John S. Pearse, University of California Santa Cruz; Dustin D. Chivers, California Academy of Sciences; and Howard M. Feder, University of Alaska, for their help and advice. Special thanks are due to those who gave field assistance both above and below the water: Lloyd Lowry, Gary M. Carmignani, Robert R. Hollis, Victor C. Norling, and James E. Norton. This project could not have been accomplished without the use of the research vessel *Octopus* owned by Oceanic Products, San Leandro, California, on which I lived and which I used as a base of operations.

SUMMARY

1. Intraspecific agonistic behavior, called bouts, which involves ray interactions between individual sea stars, is reported in species from all three living orders of asteroids and described for *Patiria miniata* and *Pycnopodia helianthoides.* This behavior indicates a certain sensitivity to conspecific contact on the aboral surface, in which each individual attempts to place a ray or rays on top of its opponent, an act which may initiate opponent withdrawal. Agonistic intraspecific bouts affect the distribution and feeding of both species although bouts may take place where food is not present.

2. *Patiria* bouts may last over two hours and sometimes terminate by individuals overlapping and sharing food. Evidence suggests that *Patiria* bouts are: (a) quite common; (b) not related to sexual behavior; (c) territorial; and (d) influenced as to outcome by relative body orientation; however, when territorial behavior is involved, relative body orientation does not affect bout outcomes. *Pycnopodia* bouts are of shorter duration (up to 10 minutes), terminating with the withdrawal of one or both animals, and sometimes resulting in an extensive pursuit.

3. Two forms of intraspecific *Patiria* bouts are noted: continuous-contact and intermittent-contact. Intermittent-contact bouts appear less intensive, permitting individuals to feed and to engage in bouts with more than one opponent at a time. It is speculated that intermittent-contact bouts are a type of ritualized activity of a fairly complex nature.

4. Interspecific bouts between specimens of *Pycnopodia* and two species of *Pisaster* (*P. giganteus* and *P. brevispinus*) have been observed only when the *Pisaster* sp. is in possession of food. *Pycnopodia* individuals approach and place

rays on the aboral surface of the feeding sea star while attempting to obtain the food with other rays. The specimen of *Pisaster* sp. actively counterattacks using its pedicellariae.

LITERATURE CITED

BLEST, A. D., 1961. The concept of 'ritualization.' Pages 102–124 in W. H. Thorpe and O. L. Zangwill, Eds., *Current Problems in Animal Behaviour.* Cambridge University Press, London.

BRANHAM, J. M., S. A. REED, J. H. BAILEY AND J. CAPERON, 1971. Coral-eating sea stars *Acanthaster planci* in Hawaii. *Science,* **172**: 1155–1157.

BRUN, E., 1969. Aggregation of *Ophiothrix fragilis* (Abildgaard) (Echinodermata: Ophiuroidea). *Nytt. Mag. Zool.,* **17**: 153–160.

DINGLE, H., 1969. A statistical and information analysis of aggressive communication in the mantis shrimp *Gonodactylus bredini* Manning. *Anim. Behav.,* **17**: 561–575.

DIX, T. G., 1969. Aggregating in the Echinoid *Evechinus chloroticus. Pac. Sci.,* **23**: 123–124.

FARMANFARMAIAN, A., A. C. GIESE, R. A. BOOLOOTIAN AND J. BENNETT, 1958. Annual reproductive cycles in four species of west coast starfishes. *J. Exp. Zool.,* **138**: 355–367.

FEDER, H. M., AND A. M. CHRISTENSEN, 1966. Aspects of asteroid biology. Pages 87–127 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience, New York.

GOREAU, T. F., 1964. On the predation of coral by the spiny starfish *Acanthaster planci* (L.) in the southern Red Sea. *Bull. Haifa Sea Fisheries Stat. No.* **35** (Rept. Israel 1962 South Red Sea Expedition No. 2): 23–26.

GREER, D. L. 1961. Feeding behavior and morphology of the digestive system of the sea star *Pycnopodia helianthoides* (Brandt) Stimpson. *Master's thesis, University of Washington,* 89 pp.

KJERSCHOW-AGERSBORG, H. P., 1922. The relation of the madreporite to the physiological anterior end in the twenty-rayed starfish, *Pycnopodia helianthoides* (Stimpson). *Biol. Bull.,* **42**: 202–216.

LANDENBERGER, D. E., 1966. Learning in the Pacific starfish *Pisaster giganteus. Anim. Behav.,* **14**: 414–418.

MAUZEY, K. P., C. BIRKELAND AND P. K. DAYTON, 1968. Feeding behavior of asteroids and escape responses of their prey in the Puget Sound region. *Ecology,* **49**: 603–619.

MENGE, J. L., AND B. A. MENGE, 1974. Role of resource allocation, aggression and spatial heterogeneity in coexistence of two competing intertidal starfish. *Ecol. Monogr.* **44**: 189–209.

NOBLE, G. K., 1939. Symposium on the individual *vs.* the species. IV. The role of dominance in the social life of birds. *Auk,* **56**: 263–273.

PEARSE, J. S. AND S. W. ARCH, 1969. The aggregation behavior of *Diadema* (Echinodermata, Echinoidea). *Micronesica,* **5**: 165–171.

REESE, E. S., 1966. The complex behavior of echinoderms. Pages 157–208 in R. A. Boolootian, Ed., *Physiology of Echinodermata.* Interscience, New York.

ROBILLIARD, G. A., 1971. Feeding behaviour and prey capture in an asteroid, *Stylasterias forreri. Syesis,* **4**: 191–195.

TINBERGEN, N., 1952. "Derived" activities; their causation, biological significance, origin, and emancipation during evolution. *Quart. Rev. Biol.,* **27**: 1–32.

WARNER, G. F., 1971. On the ecology of a dense bed of the brittlestar *Ophiothrix fragilis. J. Mar. Biol. Assn. U. K.,* **51**: 267–282.

WOBBER, D. R., 1973. Aboral extrusion of squid pens by the sea star *Pycnopodia helianthoides. Veliger,* **16**: 203–206.

WOBBER, D. R., 1974. Agonism in asteroids. *Master's thesis, San Francisco State University,* 63 pp.

WOOLF, C. M., 1968. *Principles of biometry.* D. Van Nostrand, New York, 359 pp.

INDEX

A

Actiniaria, external brooding in, 199
Aggressive behavior, in asteroids, 483
Agonism in asteroids, 483
Aiello, E. See G. B. Stefano, 141
Amino acids, nutritional role of in *Aurelia*, 117
AMP, dietary requirement in *Artemia*, 416
Analogues, juvenile hormone in *Dermestes vulpinus*, 320
Anderson, R. S., and R. A. Good. Naturally-occurring hemagglutinin in a tunicate *Halocynthia pyriformis*, 357
Annual reproductive cycle, of *Notemigonus crysoleucas*, 402
Anomalous influence of salinity, 26
Anthozoa, reproduction in, 199
Argopecten irradians, shell growth in, 472
Artemia salina, dietary AMP requirement, 416
Ascidian, hemagglutinin, 357
Asterias vulgaris, reproduction of, 461
Asteroids, agonism in, 483
Aurelia, uptake and utilization of glycine by, 117
Astropecten latespinosus Meissner, 49
Autoradiographic study of crustacean tissue, 440

B

Barnes, C. D. See J. F. Wermuth, 344
Bean seeds, water uptake, 370
Behavior, in asteroids, 483
  starfish, 68
  Turbellaria, *Neochildia fusca* n. sp., 35
Betzer, S. B., and M. E. Q. Pilson. Copper uptake and excretion by *Busycon canaliculatum* (L.), 1
Betzer, S. B., and P. P. Yevich. Copper toxicity in *Busycon canaliculatum* (L.), 16
Biochemical composition in *Thais*, 448
Biology, of *Myzobdella*, 193
  of *Neochildia fusca* n. gen., n. sp., 35
Bivalves, reproductive physiology of, 243
  shell-growth in, 472
Blackwelder, P. L. See A. P. Wheeler, 472
Blanquet, R. S., and B. Wetzel. Surface ultrastructure of the scyphopolyp, *Chrysaora quinquecirrha*, 181
Blattella germanica, molting cycle of, 259
Brachiolaria stage, lacking in *Astropecten*, 49
Bradley, B. P. The anomalous influence of salinity on temperature tolerances of summer and winter populations of the copepod *Eurytemora affinis*, 26
Branchial ventilation in fishes, 85
Brooding, external, in *Epiactis*, 199
Brown, F. A., Jr., and C. S. Chow. Non-equivalence for bean seeds of clockwise and counterclockwise magnetic motion: a novel terrestrial adaptation?, 370
Bush, L. Biology of *Neochildia fusca* n. gen., n. sp. from the northeastern coast of the United States (Platyhelminthes: Turbellaria), 35
Busycon, copper regulation in, 1
  copper toxicity in, 16

C

Callinectes sapidus, infestation in, 193
Campanularia flexuosa, effects of radiation on, 344
Cancer irroratus Say, eyestalk removal, 440
Carr, W. E. S., and S. Gurin. Chemoreception in the shrimp, *Palaemonetes pugio*: comparative study of stimulatory substances in human serum, 380
Cecropia silkworm, metamorphosis, 429
Chemoreception, in the shrimp, 380
Childia groenlandica (Levinson, 1879), 35
Childia spinosa Graff (=*groenlandica*), 35
Chow, C. S. See F. A. Brown, Jr., 370
Chrysaora, surface ultrastructure of, 181
Ciliary currents, scyphopolyps, 181
Clams, gonad development in, 243
Cnidaria, effects of radiation on, 344
Coagulation process, in *Emerita asiatica*, 286
Cochran, R. C., and F. Engelmann. Environmental regulation of the annual reproductive season of *Strongylocentrotus purpuratus* (Stimpson), 393
Cockroach, molting cycle of, 259
Cocoons, *Myzobdella lugubris*, 193
Comanthus japonica, reproductive cycle of, 219
Control, of corpora allata function, 106
Copper, regulation of in *Busycon*, 1
  tissue concentrations of, 16
  uptake from toxic concentrations, 16
Corpora allata, control of, 106
Crab larvae, depth regulation of, 333
Crinoidea, gonadal development in, 219

D

Daniels, B. A., and R. T. Sawyer. The biology of the leech, *Myzobdella lugubris*, infesting blue crabs and catfish, 193
Daylength, and *Notemigonus* reproduction, 402
de Vlaming, V. L. Effects of photoperiod and temperature on gonadal activity in

the cyprinid teleost, *Notemigonus crysoleucas*, 402

Death sites, of *Drosophila* population, 274

Dermestes vulpinus, respiratory metabolism in, 320

Destalking, effects on Y-organs of *Cancer irroratus* Say, 440

Development, of the sea-star, 49

  cycle, hard clams, 243

Dopamine, localization of, 141

Drosophila, behavior of, 274

DUNN, D. F. Reproduction of the externally brooding sea anemone *Epiactis prolifera* Verrill, 1869, 199

E

Ecdysterone, effect of on *Dermestes*, 320

Echinoderms, social behavior, 483

  gonadal development in, 219

  respiration in, 157

Echinoidea, oxygen consumption in, 157, 165

  reproductive cycles in, 165

Electrocardiograms in swimming fish, 85

Electromyograms, branchial muscles of fishes, 85

Emerita asiatica, effect of temperature on, 286

Emigration, socially-induced in *Drosophila*, 274

ENGLEMANN, F. See R. C. COCHRAN, 393

Environmental regulation, of reproductive season of *Strongylocentrotus purpuratus*, 393

Epiactis prolifera, reproduction in, 199

Eurytemora affinis, 26

Eyestalk removal, of *Cancer irroratus* Say, 440

F

Feeding behavior, in asteroids, 483

  in *Palaemonetes pugio*, 380

Food uptake by *Pinnotheres maculatus*, 60

G

Galleria mellonella, larvae, 106

Gamma-radiation, dose response effects on *Campanularia*, 344

Gastropoda, reproduction in, 448

Geographic effects, on gonad cycle in hard clams, 243

GIESE, A. C. See S. K. WEBSTER, 165

Gill ventilation, in fishes, 8

Glycine, uptake and utilization of, by *Aurelia aurita*, 117

Gonad, activity, in *Notemigonus crysoleucas*, 402

  cycle in hard clams, 243

  cycle, seasonal variation in hard clams, 243

  development in *Comanthus*, 219

Gonoduct, of *Asterias vulgaris*, 461

GONOR, J. See I. POLLS, 68

GOOD, R. A. See R. S. ANDERSON, 357

GRANGER, N. A. See F. SEHNAL, 106

GRIMMER, J. C. See N. D. HOLLAND, 219

Growth functions, effects of radiation on *Campanularia*, 344

GURIN, S. See W. E. S. CARR, 380

H

Halocynthia pyriformis, hemagglutinin, 357

Hemagglutinin, naturally-occurring, 357

Hemocyanin, in *Busycon*, 1

Hemocytes, morphology of in *Emerita asiatica*, 286

Henricia leviuscula, righting methods, 68

HERNANDORENA, A. Metabolic significance in nucleic acid metabolism and protein synthesis of dietary AMP requirement in *Artemia salina* (L.), 416

Histofluorescent localization of serotonin and dopamine, 141

Histology, of the gonoduct of *Asterias vulgaris*, 461

Histopathology, of copper in *Busycon*, 16

HODKOVA, M. See K. SLAMA, 320

HOFFMAN, D. L. See F. P. SIMIONE, JR., 440

HOLLAND, N. D., J. C. GRIMMER AND H. KUBOTA. Gonadal development during the annual reproductive cycle of *Comanthus japonica* (Echinodermata: Crinoidea), 219

Human serum, stimulatory substances in, 380

Hydrodynamics, fish ventilation and swimming, 85

Hypermetabolism, in *Dermestes*, 320

I

Ictalurus catus, infestation in, 193

Insects, hormones and bioanalogues, 303

Isotope incorporation, diurnal growth, 472

J

Juvenile hormone, covert effects of, 429

  inducing delay of metamorphosis, 429

Juvenile sea-star of *Astropecten*, 49

K

KECK, R. T., D. MAURER AND H. LIND. A comparative study of the hard clam gonad developmental cycle, 243

KOMATSU, M. On the development of the sea-star, *Astropecten latespinosus* Meissner, 49

KRUCZYNSKI, W. L. A radioactive tracer study of food uptake by *Pinnotheres maculatus* in molluscan hosts, 60

KUBOTA, H. See N. D. HOLLAND, 219

KUNKEL, J. G. Cockroach molting. I. Temporal organization of events during molting cycle of *Blattella germanica* (L.), 259

L

Larvae, of *Busycon*, copper in, 1
of *Galleria mellonella*, 106
Lectin, tunicate, 357
Leptasterias aequalis, righting methods, 68
Leptodius floridanus, influence of light on, 333
Life-history, Turbellaria, *Neochildia fusca* n. sp., 35
Light, effect on crab larvae, 333
LIND, H. See R. T. KECK, 243

M

Magnetic motion, clockwise and counterclockwise, 370
Magnets, rotating, 370
Mantle, shell, in *Argopecten*, 472
MAURER, D. See R. T. KECK, 243
Metabolic significance, of dietary AMP requirement in *Artemia salina*, 416
Metamorphosis, of *Astropecten*, 49
of the viscera of the Cecropia silkworm, 429
Methyladenine, for maturation and spawning in sea-star, 49
MILKMAN, R. Specific death sites in a *Drosophila* population cage, 274
Mineralization, molluscan, 472
Molluscs, reproductive strategies, 448
reproductive physiology of, 243
Molting cycle, of *Blatella germanica*, 259
Morphology, of the gonoduct of *Asterias*, 461
Mortality, socially-induced in *Drosophila*, 274
Muscle, of *Squalus acanthias*, 303
Mytilus edulis, nervous system of, 141
Myzobdella, seasonal abundance of, 193

N

Neochildia fusca n. gen., n. sp., biology of, 35
Nitrogenous constituents in plasma of *Squalus acanthias*, 303
Non-equivalence, magnetic rotation, for bean seeds, 370
Notemigonus crysoleucas, gonadal activity, 402
Nucleic acid metabolism, significance in, 416

O

Olfaction, effect on feeding behavior, 380
Oocytes, volume fluctuations in, 219
Oogenesis, in Crinoidea, 219
Osmolality, of *Squalus acanthias*, 303
Oxygen consumption, in echinoderms, 157, 165
in echinoids, 165
in insects, 320

P

Palaemonetes pugio, chemoreception in, 380
Panopeus herbstii, influence of light on, 333
Photic responses, of crab larvae, 333
Photoperiod, effect on gonadal activity, 402
PILSON, M. E. Q. See S. B. BETZER, 1
Pinnotheres maculatus, food uptake, 60
Plasma, of *Squalus acanthias*, 303
Platyhelminthes: Turbellaria, 35
POLLS, I., AND J. GONOR. Behavioral aspects of righting in two asteroids from the Pacific coast of North America, 68
Population, density control, of *Drosophilia*, 274
summer and winter, and thermal tolerance, 26
Prosobranchia, reproduction in, 448
Protein metabolism, in *Artemia*, 416
Pupal differentiation, and juvenile hormone, 429

R

Radiotracer copper in *Busycon*, 1
Ram ventilation, in fish gills, 85
RAVINDRANATH, M. H. Effects of temperature on the morphology of hemocytes and coagulation process in the mole-crab *Emerita* (=*Hippa*) *asiatica*, 286
Recovery, thermal shock in *Eurytemora*, 26
Regeneration, effect of on molting cycle in *Blatella*, 259
Regulation, of copper in *Busycon*, 1
Reproduction, in Crinoidea, 219
in echinoids, 165
in *Epiactis prolifera*, 199
of *Strongylocentrotus purpuratus*, 393
of *Notemigonus crysoleucas*, 402
in *Thais lamellosa*, 448
Reproductive systems of sea-stars, 461
Respiratory reflexes in swimming fish, 85
RIDDIFORD, L. M. Juvenile hormone-induced delay of metamorphosis of the viscera of the Cecropia silkworm, 429
Righting methods, in asteroids, 68
RNA synthesis, control in crustacean moltcycle, 440
ROBERTS, J. L. Active branchial and ram gill ventilation in fishes, 85
ROBERTSON, J. D. Osmotic constituents of the blood plasma and parietal muscle of *Squalus acanthias* (L.), 303
Rotation, effect on bean seed growth rate, 370

S

Salinity and temperature in *Eurytemora*, 26
SAWYER, R. T. See B. A. DANIELS, 193
Scyphopolyps, surface ultrastructure of, 181
Sea urchins, comparative respiration of, 157
SEHNAL, F., AND N. A. GRANGER. Control of corpora allata function in larvae of *Galleria mellonella*, 106
Serotonin, localization of, 141
Sevin, effects on copper, 1
Shell growth, in the scallop, 472
SHICK, J. M. Uptake and utilization of dissolved glycine by *Aurelia aurita* scyphistomae: temperature effects on the uptake

process; nutritional role of dissolved amino acids, 117
Simione, F. P., Jr., and D. L. Hoffman. Some effects of eyestalk removal on the Y-organs of *Cancer irroratus* Say, 440
Skeletal system, *Astropecten*, 49
Slama, K., and M. Hodkova. Insect hormones and bioanalogues: their effect on respiratory metabolism in *Dermestes vulpinus* (L.), 320
Spermatogenesis, in Crinoidea, 219
  in *Notemigonus*, 402
Squalus acanthias, osmotic constituents of, 303
Stefano, G. B., and E. Aiello. Histofluorescent localization of serotonin and dopamine in the nervous system and gill of *Mytilus edulis* (Bivalvia), 141
Stickle, W. B. The reproductive physiology of the intertidal prosobranch *Thais lamellosa* (Gmelin). II. Seasonal changes in biochemical composition, 448
Strongylocentrotus purpuratus (Stimpson), reproductive season of, 393
Sulkin, S. D. The influence of light in the depth regulation of crab larvae, 333
Surface, epidermal, of the scyphopolyp, 181
  gastrodermal, of scyphopolyp, 181
Swimming and gill ventilation in fishes, 85

T

Temperature, effect of on morphology in *Emerita asiatica*, 286
  effect on gonadal activity, 402
  effects on uptake process, 117
Territorial behavior, in the asteroid, *Patiria miniata*, 483
Thais lamellosa, reproductive physiology of, 448
Thermal tolerance, in *Eurytemora*, 26
Toxicity, of copper in *Busycon*, 16
Tunicate, hemagglutinin, 357
Turbellaria, biology of *Neochildia,* 35

U

Uptake of glycine by *Aurelia,* 117
Uridine uptake, by crustacean molting gland (Y-organ), 440

V

Ventilation in fishes, 85
Vitellogenesis, in *Notemigonus,* 402

W

Walker, C. W. Studies on the reproductive systems of sea-stars. II. The morphology and histology of the gonoduct of *Asterias vulgaris,* 461
Water uptake, by bean seeds, 370
Webster, S. K. Oxygen consumption in echinoderms from several geographical locations, with particular reference to the Echinoidea, 157
Webster, S. K., and A. C. Giese. Oxygen consumption of the purple sea urchin with special reference to the reproductive cycle, 165
Wermuth, J. F., and C. D. Barnes. Dose-response effects of gamma-radiation on several growth functions of *Campanularia flexuosa,* 344
Wetzel, B. See R. S. Blanquet, 181
Wheeler, A. P., P. L. Blackwelder and K. M. Wilbur. Shell growth in the scallop *Argopecten irradians.* I. Isotope incorporation with reference to diurnal growth, 472
Wilbur, K. M. See A. P. Wheeler, 472
Wobber, D. R. Agonism in asteroids, 483

Y

Y-organs, effects of eyestalk removal on, 440
Yevich, P. P. See S. B. Betzer, 16

Continued from Cover Two

4. ***Literature Cited.*** The list of references should be headed LITERATURE CITED, should conform in punctuation and arrangement to the style of recent issues of THE BIOLOGICAL BULLETIN, and must be typed *double-spaced* on separate pages. Note that citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A. Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS of those abstracted (most recent issue: November, 1972). Foreign authors, and others who are accustomed to use THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K. at £0.65 or $1.75) useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (not strictly as USASI usage, *i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.* not *J.Cell.Comp.Physiol.*)

E. We strongly recommend that more unusual words in journal titles be spelled out in full, rather than employing lengthy, peculiar "abbreviations" or new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation. Even in more familiar languages, *Z. Vererbungslehre* is preferred to *Z. VererbLehre* (WORLD LIST) or *Z. Vererbungsl.* (USASI). *Accurate and complete communication of the reference is more important than minor savings in printing costs.*

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

H. Spell out *London, Tokyo, Paris, Edinburgh, Lisbon, etc.* where part of journal title.

I. Series letters *etc.* immediately before volume number.

J. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond.; Science, N.Y.; Evolution, Lancaster, Pa.*)

K. The correct abbreviation for THE BIOLOGICAL BULLETIN is *Biol. Bull.*

5. ***Figures.*** The dimensions of the printed page, 5 by $7\frac{3}{8}$ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction. Explanatory matter should be included separately in legends as far as possible, although the axes should always be numbered and identified on the illustration itself. Figures should be prepared for reproduction as either line-cuts or halftones; no other methods will be used. Figures to be reproduced as line-cuts should be drawn in black ink on white paper, good quality tracing cloth or plastic, or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on board, and both designating numbers or letters and scale-bars should be affixed directly on the figures. We recommend that halftones submitted to us be mounted prints made at about $1\frac{1}{2}$ times the linear dimensions of the final printing desired (the actual best reductions are achieved from copy in the range from $1\frac{1}{4}$ to 2 times the linear dimensions). As regards line-blocks, originals can be designed for even greater reductions but are best in the range $1\frac{1}{2}$ to 3 times. *All* figures should be numbered in consecutive order, with no distinction between text and plate-figures. The author's name should appear on the reverse side of all figures, and the inked originals for line-blocks must be submitted for block-making.

6. ***Mailing.*** Manuscripts should be packed flat. All illustrations larger than $8\frac{1}{2}$ by 11 inches must be accompanied by photographic reproductions or tracings that may be folded to page size.

Reprints. Reprints may be obtained at cost; approximate prices will be furnished by the Managing Editor upon request.

CONTENTS

ANDERSON, ROBERT S. AND ROBERT A. GOOD
Naturally-occurring hemagglutinin in a tunicate *Halocynthia pyriformis* 357

BROWN, FRANK A., JR. AND CAROL S. CHOW
Non-equivalence for bean seeds of clockwise and counterclockwise magnetic motion: a novel terrestrial adaptation? 370

CARR, WILLIAM E. S. AND SAMUEL GURIN
Chemoreception in the shrimp, *Palaemonetes pugio:* comparative study of stimulatory substances in human serum 380

COCHRAN, ROGER C. AND FRANZ ENGELMANN
Environmental regulation of the annual reproductive season of *Strongylocentrotus purpuratus* (Stimpson) 393

DE VLAMING, VICTOR L.
Effects of photoperiod and temperature on gonadal activity in the cyprinid teleost, *Notemigonus crysoleucas* 402

HERNANDORENA, A.
Metabolic significance in nucleic acid metabolism and protein synthesis of dietary AMP requirement in *Artemia salina* (L.) 416

RIDDIFORD, LYNN M.
Juvenile hormone-induced delay of metamorphosis of the viscera of the Cecropia silkworm 429

SIMIONE, FRANK P., JR. AND DANIEL L. HOFFMAN
Some effects of eyestalk removal on the Y-organs of *Cancer irroratus* Say 440

STICKLE, WILLIAM B.
The reproductive physiology of the intertidal prosobranch *Thais lamellosa* (Gmelin). II. Seasonal changes in biochemical composition 448

WALKER, CHARLES WAYNE
Studies on the reproductive systems of sea-stars. II. The morphology and histology of the gonoduct of *Asterias vulgaris* 461

WHEELER, ALFRED P., PATRICIA L. BLACKWELDER AND KARL M. WILBUR
Shell growth in the scallop *Argopecten irradians*. I. Isotope incorporation with reference to diurnal growth 472

WOBBER, DON R.
Agonism in asteroids 483

WH 1B1K $